THE BRITISH JOURNAL

OF

EXPERIMENTAL
PATHOLOGY

THE BRITISH JOURNAL OF

EXPERIMENTAL

PATHOLOGY

VOLUME THIRTY

1949

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No 6 —DECEMBER

RIFT VALLEY FEVER, THE NEUROTROPIC ADAPTATION OF THE VIRUS AND THE EXPERIMENTAL USE OF THIS MODIFIED VIRUS AS A VACCINE

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IN 1944 Rift Valley fever virus was isolated from 6 different lots of mosquitoes captured in uninhabited forest in western Uganda during investigations, the objective of which was to determine the forest vector of yellow fever (Smithburn, Haddow and Gillett, 1948) In the hope of demonstrating the presence of yellow fever virus in wild-caught mosquitoes, suspensions of the insects were inoculated intracerebrally into mice The 6 strains isolated were not immediately recognized as Rift Valley fever virus Although histological studies in a second passage mouse gave a clue to the identity, and although it was found that the agents were pathogenic by extraneural inoculation, these 6 and 2 other strains recovered from persons infected in the laboratory were transmitted by intracerebral passage, simply because of technical facility All the strains so passed in series ultimately lost their hepatotropism and retained their neurotropic properties Controlled experiments were then undertaken to determine whether this modification of the virus could be brought about regularly by intracerebral passage When this was found to be the case, studies were made to determine the efficacy of the modified (neurotropic) virus as an immunizing agent The results of these investigations are the subject of the present communication

Mackenzie and Findlay (1936) and Mackenzie, Findlay and Stern (1936) found that the neurotropic properties of Rift Valley fever virus could be retained or enhanced, with simultaneous diminution of the hepatotropism, by serial intracerebral passage of the virus in mice previously injected intraperitoneally with specific Rift Valley fever immune serum The experiments here reported indicate that the use of the immune serum is not necessary, although it may expedite the modification of the virus

MATERIALS AND METHODS

All of the strains of virus used in this investigation and most of the methods employed in studying them have been described elsewhere (Smithburn, Haddow and Gillett, 1948) Special methods employed in the present work are given here

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Titrations of virus content of tissues

Tissues were removed aseptically and weighed, and 10 per cent suspensions were prepared in 10 per cent non-immune serum in physiological saline (hereafter referred to as serum-saline)   All suspensions for use in a given test were centrifuged simultaneously for 20 minutes at approximately 3000 r p m   Serial decimal dilutions of the supernate from each suspension were prepared in serum-saline   The same flask of diluent was used for all the tissues in any one experiment   Groups of 6 or 12 mice were inoculated with each dilution of each suspension   Preparation of the dilutions was timed to coincide with the rate of inoculations   All inoculations were done by the same technician   End-points were calculated by the method of Reed and Muench (1938)

Use of modified virus as a vaccine

In mice —All the mice to be used in a given experiment were brought into the laboratory at the same time, and separated into test and control groups which were as nearly alike as possible in age and sex   The mice of one group were then inoculated intraperitoneally, each with 0 06 ml of a 1 per cent brain suspension of mice infected with intracerebral-passage virus (Smithburn, Haddow and Gillett, 1948), strain 2   At selected intervals after the immunizing injections, intraperitoneal titrations of fully virulent pantropic virus were made in both the control and vaccinated mice, using 12 mice per group in each instance

In lambs —European-native hybrid stock lambs were used   Prior to the experiments a protection test was done on each to make certain it was non-immune   In each test the immunized and control animals were as near the same age as possible, and in one instance they were twins   Single injections of 1 0 ml of 1 per cent or 10 per cent mouse-brain supernate were given subcutaneously   Following the immunizing injections, each lamb was bled daily, and its serum was inoculated intracerebrally into mice to test for the presence of the neurotropic virus in the blood   Protection tests were also made at various intervals after the injection of neurotropic virus to learn whether antibody was demonstrable in the serum   At an appropriate interval following the inoculation of neurotropic virus, the test and control lambs were inoculated subcutaneously with a challenge dose of virulent pantropic Rift Valley fever virus   Daily tests for circulating virus were made in both the test and control animals   Their sera were, in this case, inoculated intraperitoneally into mice   Temperatures were taken on lambs once daily on Sundays and holidays, and twice daily at other times   All lambs remaining alive at the end of the experiments were bled, and protection tests were done on their sera

EXPERIMENTS AND RESULTS

The first indication that our stock intracerebral-passage virus had become modified was obtained when it was noted that the titre of virus in intraperitoneal protection tests was lower than formerly, that those mice which sickened and died did so only after prolonged incubation periods, and that there was a considerable incidence of paralysis among mice inoculated either intraperitoneally or intracerebrally   Paralysis had not been observed at all in the earliest passages, even when mice were inoculated intracerebrally   In fact, a striking feature was the paucity or total absence of lesions in the brains of mice

which received intracerebral inoculations with low passage virus The pathogenic effects were manifest wholly or principally in the abdominal visceral tissues, notably in the liver By contrast, histological examinations of mice receiving high intracerebral-passage virus revealed a complete absence, or striking diminution in the extent, of hepatic lesions, and the appearance of necrosis in nerve cells in the brains of the mice

Virus lines of low, medium and high intracerebral passages were on hand at this time These were employed in experiments to determine the concentration of virus in various tissues at different passages, and to learn whether there were marked quantitative differences in the potency of virus introduced by different routes The tests with low, medium and high passage virus were made on different days, within a 2-week period In each experiment 2 sick mice were sacrificed, and titrations were made of the virus content of the pooled hearts, lungs, spleens, kidneys, livers and brains

As shown in Table I, the low passage virus was present in high concentration in all the tissues, possibly owing to its presence in the blood of the mice The

TABLE I —*Results of Intracerebral and Intraperitoneal Titrations of Virus Content of Various Tissues of Mice Inoculated with Low, Medium or High Passage Mouse-brain Virus*

Tissue	Route of inoculation	Titre of virus in tissue, 1 in—		
		9th passage	Medium* passage	79th passage
Heart	Intracerebral	31,700,000	616,000	< 10
Lung	,,	72,500,000	427,000	1,380
Spleen	,,	72,500,000	850,000	537
Kidney	,,	49,000,000	575,000	42
Liver	,,	83,000,000	31,700,000	100
,,	Intraperitoneal	16,300,000	15,800,000	< 10
Brain	Intracerebral	252,000	1,520,000	16,300,000
,,	Intraperitoneal	16,300	10,300	370

* 9th intracerebral passage of virus recovered from a human who was suffering from a laboratory infection probably induced by 22nd intracerebral passage of stock virus strain 2, isolated from mosquitoes This probably represents 31st mouse passage with one intervening human passage

concentration of virus was highest in the liver and lowest in the brain The intraperitoneal titration of the brain gave a lower value than did the intracerebral titration, a difference which is probably significant, in view of the fact that each dilution was tested by each route in groups of 12 mice

The medium passage virus showed significantly lower titres in all tissues except liver and brain The values for liver were actually less, but probably not significantly so The intracerebral titration of medium passage brain gave a higher value than did the low passage virus, but the intraperitoneal titration of medium passage brain showed a relative, if not an absolute, decline in potency

The 79th passage mouse tissues showed consistently and significantly lower virus titres in every instance except in the brain titrated intracerebrally The latter tissue showed a significant rise in virus content However, comparison of the intracerebral and intraperitoneal titrations reveals that, although the brains contained a great deal of virus, it was not very effective when introduced intraperitoneally

These studies made it obvious that the virus had undergone a progressive modification during the course of serial intracerebral passages It seemed

important to determine whether this was an accidental phenomenon, or whether
it occurred regularly, and could be induced under controlled conditions
Accordingly, an ampoule of desiccated 7th intracerebral-passage virus, known
to possess high hepatotropic potency, was rehydrated, and groups of mice were
inoculated intracerebrally and intraperitoneally Employing brain tissue,
serial intracerebral passages were made from the group inoculated intracerebrally ,
and from the group inoculated intraperitoneally serial intraperitoneal passages
of liver suspension were made   Tissues of mice in each of these series were
studied histologically at various passage intervals   Further, intracerebral
and intraperitoneal titrations of 9th intracerebral-passage mouse livers and
brains were made, and intracerebral and intraperitoneal titrations of livers and
brains of both passage lines were made at the 20th and 61st transfers   The
titrations of 9th-passage mouse tissues formed a part of the previous experiment.

The results of these tests, shown in Table II, indicate that the decline in
hepatotropism with serial intracerebral passage of Rift Valley fever virus was

TABLE II —*Results of Tests Showing Loss of Hepatotropism and Enhancement
of Neurotropism with Serial Intracerebral Passage, and Maintenance of
Hepatotropism with Passage of the Same Strain Intraperitoneally*

Passage line	Tissue tested	Route inoculated	Titre of virus, 1 in—		
			9th passage	20th passage	61st passage
Intracerebral passages employing brain tissue	Brain	Intracerebral	252,000	56,000	1,580,000
		Intraperitoneal	16,300	14,200	< 10*
	Liver	Intracerebral	83,000,000	31,700,000	32
		Intraperitoneal	16,300,000	1,420,000	< 10*
Intraperitoneal passages employing liver tissue	Brain	Intracerebral		6,900	56,000
		Intraperitoneal		5,000	100,000
	Liver	Intracerebral		31,700,000	317,000,000†
		Intraperitoneal		23,000,000	317,000,000†

* 10 per cent suspension caused neither illness nor death
† End points not obtained

not an accidental phenomenon   It occurred again and under controlled
conditions   There was, in addition, evidence of simultaneous enhancement
of neurotropism with serial intracerebral passage   On the other hand, hepato-
tropism (or pantropism) was well maintained by intraperitoneal transfer of
liver tissue made through an equal number of passages and over the same period
of time

Histological studies

Tissues from mice of the earliest passages, at which time paralysis was not
a part of the clinical picture, exhibited the following features
Brain —Neither gross nor microscopic lesions attributable to the virus were
present
Liver —This tissue was the site of the principal lesions   Usually the liver
was brownish red in colour and less firm than normal, but not increased in size
Congestion was intense and haemorrhages were common   The hepatic cells
were involved in a pan-lobular necrosis of extreme degree   Many hepatic cells
showed swollen, vesicular nuclei with chromatin arranged around the nuclear
membranes , some cells so affected contained intranuclear acidophilic inclusions

of round, oval or irregular form   Other cells exhibited pyknosis or karyorrhexis
Nuclear debris was abundant   Less affected cells showed only cloudy swelling,
but very few hepatic cells escaped injury   The hepatic cords were disrupted
Small, round or oval, brightly acidophilic " hyaline pearls " were scattered
through the tissues, some apparently in sinusoids, others in the cytoplasm of
hepatic cells   In the occasional mice which survived longer than the average,
the debris of the mass of necrotic cells was in the process of being engulfed by
large, highly vacuolated phagocytes, probably Kupffer cells

A systematic study of the progressive changes occurring during the neuro-
adaptation of Rift Valley fever virus has been made by S F Kitchen   It
suffices here to record the lesions found in high intracerebral-passage mice, and
to compare these with the lesions induced by the early-passage pantropic virus

Liver —Tissue from a mouse of the 67th passage showed minimal evidence
of the lesions described above   No hepatic lesions were observed in 78th- and
81st-passage mice

Brain —The principal lesions in mice of the 67th, 78th and 81st intracerebral
passages were in the brain   These consisted of congestion, diffuse necrosis of
ganglion cells with derangement of their chromatin pattern (in the 67th passage,
most pronounced in Ammon's horn) and slight diffuse infiltration

Thus, concurrently with the loss of virulence by intraperitoneal inoculation,
the intracerebral-passage virus had lost all, or nearly all, its capacity to produce
lesions in the liver, and had acquired the ability to produce lesions in the brain

Experiments on Immunization with the Neurotropic Virus

The pathogenicity of the virus for abdominal visceral tissues had been so
reduced by continued brain-to-brain passage that intraperitoneal introduction
of the agent seldom resulted in death   Consequently, it was decided to study
its capacity to induce an effective immunity   Two tests were made in mice
and 4 in lambs

Tests of immunizing potency in mice

The first experiment in mice employed 4 groups of vaccinated animals and
2 groups of non-vaccinated controls   One group of mice received 2 intraperitoneal
immunizing injections of the neurotropic virus spaced by an interval of 1 week
Three other groups of mice received single intraperitoneal injections of the same
virus   The vaccinating dose was 0 06 ml of 1 per cent brain suspension   The
virus used on the first occasion was the unfiltered supernate of an 83rd intra-
cerebral-passage mouse brain suspension   That used on the second occasion
was the Seitz filtrate of an 85th intracerebral-passage mouse brain suspension
Twelve mice out of the 401 in the 4 vaccinated groups died in the interval of
3 weeks between the first injections and the challenge inoculations   Two out
of 201 unvaccinated controls died in the same period

Three weeks after the first and 2 weeks after the last immunizing injections
all the surviving vaccinated and control mice were inoculated intraperitoneally
with pantropic virus   The challenge inoculum was a Seitz filtrate of a suspension
of the livers of 2 sick mice of the 62nd intraperitoneal liver-passage group   In
order to determine the average survival time, 22 to 28 mice of each vaccinated
or control group were given intraperitoneal injections of 0 06 ml of 1 in 100 000

liver suspensions   The remainder of the mice in each group were used for titration of the challenge virus, with 12 of each group receiving each virus dilution Results of the test are shown in Table III

TABLE III —*Results of Challenge Inoculations with Pantropic Rift Valley Fever Virus in First Experiment on Immunization of Mice with the Neurotropic Virus*

Group	Date born	Date vaccinated	Sex.	Virus dilution, log	Fate of mice Died *	Fate of mice Lived	Titre of virus, 1 in—	Average survival time (days)
A	1–3 July	12 August	F	4	0	12	< 10,000	10+
			F	5	0	12		
			F	6	0	11		-
			F	7	0	12		
			F	8	1	11		
			3F , 9 M	9	0	11		
			M	5	0	22		
B	1–3 ,,	12 ,,	F	4	0	8	< 10,000	10+
			F	5	0	8		
			F	6	0	6		
		19 ,,	F	7	1	7		
			F	8	0	12		
			7F , 5M	9	0	11		
			M	5	0	28		
C	1–3 ,,	19 ,,	F	4	0	12	< 10,000	10+
			F	5	0	12		
			F	6	0	12		
			F	7	0	12		
			11F , 1M	8	0	12		
			M	9	0	12		
			M	5	0	23		
D	9 ,,	19 ,,	F	4	0	12	< 10,000	10+
			F	5	0	12		
			F	6	0	12		
			F	7	0	12		
			4F , 8M	8	0	12		
			M	9	0	12		
			M	5	0	27		
E	1–3 ,,	Controls, not vaccinated	F	4	12	0	56,000,000	1 81
			F	5	12	0		
			F	6	12	0		
			F	7	12	0		
			F	8	2	10		-
			6F , 6M	9	3	9		
			M	5	27	0		
F	9 ,,	Ditto	F	4	12	0	32,400,000	2 57
			F	5	12	0		
			F	6	11	1		
			F	7	10	2		
			11F , 1M	8	3	9		
			M	9	0	12		
			M	5	26	2		

* Deaths in the first 24 hours are disregarded as being probably due to causes other than specific infection

In this experiment the protection afforded mice by the immunizing injections of neurotropic virus was nearly absolute   Considering the 2 control series of mice, E and F, as one group, the maximal test dose of virus was 4380 LD_{50}

The vaccinated mice of groups A, C and D resisted this and smaller doses completely The test dose of virus in the groups used for determination of the mean survival time was 438 LD_{50} There were only 2 survivors out of the 55 non-vaccinated mice of series E and F used for this purpose The average survival time was 2 2 days By contrast, there were no deaths among the 100 mice of the 4 vaccinated groups receiving the same inoculum

There were 20 deaths among the 101 mice of series B within the first 24 hours after challenge inoculation (these are not shown in Table III as they were not caused by the specific pathogenic action of the virus), whereas there were only 2 deaths in the same period among the remaining 369 mice which received the challenge inoculation The mice of series B were the only ones which received more than 1 immunizing injection, and these early deaths following the challenge inoculation may have been due to allergic reactions An experiment was subsequently done to test this possibility , although the result gave indication that hypersensitiveness was involved, the point was not proved beyond doubt

Having found that mice are protected against challenge inoculation with pantropic virus within 2 weeks after a single intraperitoneal injection of the relatively innocuous (by this route) neurotropic virus, a second experiment was done to determine how early this protection is-manifest A large group of mice was inoculated intraperitoneally, each with 504,000 intracerebral LD_{50} of a Seitz-filtered 1 per cent mouse brain suspension of the 83rd intracerebral passage Although the virus used to immunize in this experiment was of the same passage as that used in the first experiment in mice, it was of a different line, which was likewise derived from desiccated 7th passage virus, and likewise observed to have undergone a similar neurotropic modification with continued intracerebral passage in series

The neurotropic virus used in this experiment was more lethal by intraperitoneal inoculation than that used in the first experiment Thirty-three of the 348 mice receiving it died as a result ; only 1 of 313 unvaccinated controls died during the same period In addition, there were, among the 6 series comprising 315 vaccinated mice, 14 scattered deaths following challenge inoculation Some of these may have been caused by the neurotropic virus The mortality was thus about 3 times as high in this series of vaccinated mice as in the first series

At intervals of 4, 7 and 10 days after the immunizing injections of neurotropic virus, pantropic virus was titrated in groups of 6 vaccinated and 6 control mice Fourteen days after the single immunizing injections, pantropic virus was titrated intraperitoneally in groups of 12 vaccinated and 12 normal mice , neurotropic virus was also titrated intracerebrally in both vaccinated and normal mice At the same time, special groups were also inoculated with each virus to determine the average survival time

The results of this experiment are shown in Table IV, which indicates that the intraperitoneal inoculation of mice with neurotropic virus protects these animals within 4 days against inoculation with pantropic virus The most concentrated dilution of virus inoculated in the 4th-day test contained 31,700 LD_{50} of virus, yet there were no deaths among the vaccinated mice receiving this dose of challenge inoculum Similar results were obtained in the 7th, 10th and 14th day post vaccination challenges with pantropic virus

The fact of induced resistance is further emphasized by the data on average

TABLE IV —*Condensed Protocol of Results of the Second Experiment on Immunization of Mice with Neurotropic Rift Valley Fever Virus, followed by Challenge Inoculations with Pantropic and Neurotropic Virus*

Vaccinated or controls	No of mice per group	Interval between vaccination and challenge (days)	Challenge virus	Route of inoculation of challenge virus	Titre challenge virus, 1 in—	Average survival time (days) *
Vaccinated	6	4	Pantropic	Intraperitoneal	<10,000	
Controls					317,000,000	
Vaccinated	6	7	,,	,,	< 10,000	
Controls					142,000,000	
Vaccinated	6	10	,,	,,	< 10,000	
Controls					1,000,000,000	
Vaccinated	12	14	,,	,,	< 10,000	10+ (17,800)†
Controls					178,000,000	1 06 (17,800)
Vaccinated	12	14	Neurotropic	Intracerebral	14,800	9 26 (100)
Controls					1,000,000	3 51 (100)

* Based on 29 to 33 mice per test

† Figures in parentheses indicate LD_{50} of virus inoculated for the test of average survival time

survival time in vaccinated and control mice challenged with either pantropic or neurotropic virus, and by the results of titrations of neurotropic virus in vaccinated and control mice   The latter result is especially significant in view of the fact that the intracerebral challenge with neurotropic virus is a very severe test

Tests of immunizing potency in lambs

Two lambs were used in the first experiment   When these animals were obtained they were said to be about 5 months old   They had been in our hands 3 months when these experiments were done   In view of the result in Lamb 5, referred to later, it seems highly probable that this one, at least, was somewhat older than stated   Lamb 4 was inoculated subcutaneously with 1 0 ml of unfiltered 1 per cent mouse brain suspension of the 83rd intracerebral passage of Rift Valley fever virus   On the 21st day following the inoculation with neurotropic virus, this animal and the unvaccinated control, Lamb 5, were each inoculated subcutaneously with 1 0 ml of 1 in 100,000 dilution of pantropic Rift Valley fever virus (61st intraperitoneal liver passage in mice)   The dose for each lamb proved to be 7314 intraperitoneal LD_{50}

All the mice inoculated intracerebrally with serum taken from Lamb 4 during a period of 10 days after inoculation with the neurotropic virus survived   Therefore there was no demonstrable circulating virus   Lamb 4 had irregular temperatures prior to this inoculation, once as high as 105 6° F , and on several occasions up to 104 6° F   On the morning following the injection of the neurotropic virus its temperature was 104 6° F , and on the 7th day its afternoon temperature was 105 0° F   On 6 other occasions during the 10-day period after inoculation with the neurotropic virus its temperature was between 104 0 and 104 6° F.   It is not clear whether this represented a febrile reaction or not,

but if so the reaction was not marked   The animal continued to eat normally and at no time appeared ill

Table V shows the mortality ratios in mice used to test the sera of lambs 4 and 5 for circulating pantropic virus on the 2nd to 10th days following the challenge inoculation

TABLE V

Days after challenge	Lamb 4 (vaccinated)	Lamb 5 (normal control)
2	0/6	6/6
3	0/6	6/6
4	0/6	6/6
5	0/6	0/6
6	0/6	0/6
7	0/6	0/6
8	0/6	0/6
9	0/6	0/6
10	0/6 ‾	0/6

Numerator indicates number of mice which died , the denominator indicates the number inoculated

No tests for circulating virus ,were made on the first day after inoculation with pantropic virus   Lamb 5, the non-vaccinated control, showed circulating virus from the 2nd to 4th day, but none thereafter   All tests on the vaccinated lamb were negative

Lamb 4, the vaccinated animal, had temperatures of 105 0° and 105 6° F on the afternoons of the 1st and 4th days respectively after inoculation with pantropic virus, but its temperature was within normal limits at other times during the 10-day observation period   It is possible that this represents a mild saddleback elevation   However, the temperatures in this animal were so variable that it is not certain that it had a febrile response   Lamb 5, on the other hand, had a fairly stable preinoculation temperature record, with a maximum normal of 104 2° F   Forty-eight hours after inoculation with pantropic Rift Valley fever virus its temperature rose sharply to 106 2° F   Its temperature was normal the next morning, and remained so   It is noteworthy that, although the animal was afebrile on the 3rd and 4th days after inoculation, it had circulating pantropic virus on both those days

Results of the protection tests on Lambs 4 and 5, shown in Table VI, demonstrate that Lamb 4 developed protective humoral antibody following inoculation with the neurotropic virus, and that it had a further rise in antibody following inoculation with the pantropic virus   Whether the antibody response to the neurotropic virus was submaximal, or whether the interval between the 2 inoculations did not permit a complete response to the neurotropic before inoculation of the pantropic virus, cannot be determined from this result   In other experiments, however, we have found evidence to indicate that the neurotropic virus is a less efficient antigen than the pantropic   Nevertheless, that it does induce antibody formation is shown by the results in Table VI

An interesting incidental observation was made during this experiment Unknown to us, Lamb 5 was pregnant at the time of the experiment   On the

TABLE VI —*Results of Intraperitoneal Rift Valley Fever Protection Tests on Sera of a Vaccinated Lamb at Various Intervals after Inoculation with Neurotropic Virus, and on Sera of This and the Control Lamb after Challenge Inoculation with Pantropic Virus*

Serum	Days after vaccination	Days after challenge inoculation	Test virus dilution, log	Fate of mice		Titre of virus, 1 in—
				Died	Lived	
Lamb 4, preinoculation	0		5	6	0	31,700,000
			6	5	1	
			7	4	2	
			8	2	4	
			9	1	5	
August 22	10		5	6	0	2,520,000
			6	5	1	
			7	0*	6	
September 2	21		5	6	0	1,000,000
			6	3	3	
			7	0	6	
„ 11	30	9	5	2	4	< 100,000
			6	0	6	
„ 19	38	17	5	2	4	< 100,000
			6	0	6	
Lamb 5, preinoculation			5	6	0	23,000,000
			6	6	0	
			7	4	2	
			8	1	5	
			9	0	6	
September 10	-	8	5	6	0	317,000
			6	0	6	
„ 19		17	5	0	6	< 100,000
			6	0	6	
Lamb 3, immune control			2	1	5	< 100
			3	0	6	

    * Additional tests with higher dilutions beyond the end-points with this and other sera are omitted

7th day after inoculation she aborted a nearly full-term foetus This animal was said to be about 5 months old when it was received It had been in our possession 3 months when this experiment was done Little is known about the age of maturity of native or European-native hybrid sheep, but in view of its pregnancy it seems probable that Lamb 5 was older than we were led to believe Serum and a suspension of liver from the foetus were inoculated into mice Virus was recovered from both, and in each instance was identified by a protection test as Rift Valley fever virus The most interesting point in this connection is that the virus which traversed the placenta was present in both the blood and the liver of the foetus 72 hours following the last positive test on the serum of the mother

In the second experiment one weaned (No 14) and 2 unweaned (No 13 and 15) lambs were used as controls Another weaned (No 16) and 2 unweaned (No 11 and 12) lambs were inoculated subcutaneously with 83,916,000 mouse-intracerebral LD_{50} of 83rd intracerebral-passage virus All the tests for circulating virus were negative, showing that there was no demonstrable neurotropic virus in the blood of either of the lambs during the 14 days following inoculation Lamb 11 had no temperature reaction whatever to the inoculation of neurotropic virus Lamb 12 had a temperature of 104 3° F 30 hours after vaccination and normal temperatures at other times Normal maximum for this lamb was 103 4° F Weaned Lamb No 16 had temperatures of 104 3° F

in the first 3 days after the inoculation, and normal temperatures thereafter These reactions probably represent mild febrile responses in Lambs 12 and 16

Three weeks after the immunizing injections these animals, together with their non-vaccinated controls, were inoculated subcutaneously, each with 2,922,500 mouse-intraperitoneal LD_{50} of pantropic virus The non-vaccinated lambs showed virus in their sera for 2, 3 and 4 days, but not thereafter, while the vaccinated lambs gave consistently negative results, as may be seen from Table VII

TABLE VII —*Mortality Ratios in Mice Inoculated with Sera of Lambs*

Day after inoculation	Vaccinated lambs, No			Normal lambs, No		
	11	12	16	13	14	15
1	0/6	0/6	0/6	6/6	6/6	6/6
2	0/6	0/6	0/6	6/6	5/6	6/6
3	0/6	0/6	0/6	6/6	0/6	5/6
4	0/6	0/6	0/6	6/6	0/6	0/6
5	0/6	0/6	0/6	0/6	0/6	0/6
6 to 10	All tests negative for virus					

Two of the vaccinated lambs (No 11 and 12) had no febrile reactions following inoculation with the pantropic virus Lamb 16 had a temperature of 104 4° F on the 7th day following inoculation, but had normal temperatures at other times Each of the 3 non-vaccinated lambs had a sharp temperature reaction of 1 to 4 days' duration The peaks, 106 2° to 107 6° F , in each instance occurred on the second post-inoculation day

All the control lambs in this experiment survived The demonstration of the immunizing action of the neurotropic virus therefore depended on the temperature reactions, the tests for circulating virus, and the protection tests with serum specimens taken prior to the challenge inoculation with pantropic virus

In Table VIII, showing results of the protection tests, it is obvious that Lambs 11, 12 and 16 had specific antibody responses to the inoculation of neurotropic virus Lambs 11 and 16 exhibited further rises in antibody titre following challenge inoculation with the pantropic virus Each of the control lambs likewise had protective antibody in its serum 14 days after injection of the pantropic virus

Up to this point the experiment was wholly successful, and gave complete confirmation of the first experiment It shows that the neurotropic virus is efficacious as a vaccine, and that it is not followed by severe reactions, nor by the appearance of a demonstrable quantity of the neurotropic virus in the circulation However, the fact that the 3 vaccinated lambs died from undiscovered cause on the 8th, 25th and 33rd days after challenge inoculation with pantropic virus marred the experiment Prior to death each animal became very weak, had subnormal temperature and passed large bulky stools There was no paralysis, and the animals were able and willing to eat when food was placed before them Subinoculation of blood and suspensions of their brains to mice yielded nothing Gross autopsies and microscopic examinations failed to show a cause of death The only lesions of consequence were moderate degenerative changes in the renal tubules, the presence of albuminous deposits in their lumina and intestinal

TABLE VIII —*Results of Intraperitoneal Rift Valley Fever Protection Tests Showing Development of Protective Antibody in Lambs of the Second Experiment*

| Lamb No | Serum, taken | | | Titre of virus, 1 in— |
	Date	Days after neurotropic	Days after pantropic	
5 normal control	2 Sept , 1946			100,000,000
11	13 Nov , premoculation			25,200,000*
11	6 Dec	21		560,000
11	20 ,,	35	14	< 100,000†
12	13 Nov , premoculation			31,700,000*
12	6 Dec	21		< 100,000†
12	13 ,,	28	7	252,000
16	13 Nov , premoculation			31,700,000*
16	6 Dec	21		2,520,000
16	20 ,,	35	14	< 100,000†
13	6 ,, premoculation			31,700,000*
13	20 ,,		14	< 100,000†
14	6 ,, premoculation			6,900,000*
14	20 ,,		14	100,000
15	6 ,, premoculation			31,700,000*
15	20 ,,		14	< 100,000†
3 immune control	1 July, 1946			100

* 1 in 10,000,000 virus was the weakest dilution inoculated and complete end points were not obtained

† 1 in 100,000 virus was the strongest dilution inoculated and serum was protective against this

worms in 2 of the animals   During the experiment all the lambs were kept in an insect-proof room with cement floor and were fed solely on grass   It is possible that the 3 lambs succumbed from inanition, as the vaccinated animals were bled daily for 2 weeks longer than the controls   The cause of death was not discovered There was no evidence that either the neurotropic or the pantropic virus was responsible for the fatal issue   In fact, each of the animals had protective antibody in its serum prior to inoculation with the pantropic virus, and exhibited little or no reaction to that agent

Two sets of twin lambs were available for two further experiments on immunization   However, the first test could not be completed, because the control lamb died from undiscovered cause prior to challenge inoculation

The twin Lambs 19 and 20 were born March 30, 1947, to a non-immune ewe   Five days later both were bled for protection tests, and Lamb 19 was inoculated subcutaneously with 67,200,000 mouse-intracerebral LD_{50} of 98th-passage neurotropic Rift Valley fever virus   The lamb was bled daily for 10 days from the jugular vein, and its whole blood was inoculated intracerebrally into mice to test for circulating virus   All the inoculated mice remained well, so no evidence was obtained that the virus entered the blood   The lamb had no temperature reaction to the inoculation and exhibited no objective signs A protection test made on serum taken from this animal 21 days after inoculation of the neurotropic virus showed that it had developed protective antibody as a result of the procedure   Its twin, intended for use as control for the challenge inoculation, having died, the test inoculation of Lamb 19 was not done   Nevertheless, the experiment showed that new-born lambs may be safely inoculated with the neurotropic virus, and that they develop neutralizing antibody as a result

Lambs 21 and 22 were also twins born to a non-immune ewe   When they were 4 days old both were bled for protection tests, and No 21, a male, was

inoculated subcutaneously with 1,781,000 mouse-intracerebral LD_{50} of 100th-passage neurotropic virus   Lamb 22, a female, was reserved as the control for the challenge inoculation

The tests for circulating virus in Lamb 21 in the 10 days following inoculation with neurotropic virus were negative for that virus   However, another agent pathogenic for mice but not neutralized by potent Rift Valley fever immune serum was present in the blood of the lamb for the first 4 days   This agent caused death in mice in 5 to 7 days , it was not identified   Lamb 21 had no elevation of temperature during the 3 weeks following the inoculation of neurotropic Rift Valley fever virus   Neither the latter nor the unidentified agent in its circulation which was pathogenic for mice sufficed to induce a febrile reaction   Serum taken from Lamb 21 on the 18th day after the immunizing injection contained abundant neutralizing antibody against pantropic Rift Valley fever virus

On the 39th day after the inoculation of Lamb 21 with the neurotropic virus, Lambs 21 and 22 each received subcutaneously a challenge inoculation of 158,500 mouse-intraperitoneal LD_{50} of pantropic Rift Valley fever virus   The vaccinated lamb had no temperature exceeding 104 0° F following the challenge inoculation, and daily tests for circulating virus were negative   The non-vaccinated twin control, Lamb 22, had a sharp rise in temperature to 105 0° F 48 hours after inoculation, but its temperature was normal the following day, and remained so   The tests in mice for circulating virus in Lamb 22 showed that Rift Valley fever virus was present in the blood during the first 72 hours after the test inoculation   Thirteen days after the inoculation with pantropic virus the sera of Lambs 21 and 22 both gave complete neutralization in tests against 560,000 LD_{50} of pantropic virus

Inherited Immunity Against Rift Valley Fever

Daubney and Hudson (1931) pointed out that very young lambs are much more susceptible to Rift Valley fever than older ones   In order to protect lambs by active immunization through their period of maximal susceptibility, it would be necessary to inoculate them very soon after birth   This might not only be impractical, but might also be associated with some hazard   Another approach to the problem would be to immunize the ewes, provided they passed on the immunity to their offspring, and provided the inherited immunity were sufficiently enduring to be of practical importance

During the course of our investigations an opportunity was afforded to study the duration of the humoral immunity inherited by lambs born to an immune ewe   On December 31, 1946, Rift Valley fever immune Ewe No 1 delivered twin male lambs which were given numbers 17 and 18   Fifteen days after they were born the sera of these lambs were both found to be protective against pantropic Rift Valley fever virus   When they were 1 month old Lamb 17 was taken from the ewe and fed thereafter on cows' milk and grass   Lamb 18 remained with the mother , it fed on grass and continued to suckle   Each animal was bled once a week from the time they were 38 days old until they were 171 days old, and protection tests were done on their sera

The data on these tests were very voluminous, and only a selected few are presented in Table IX   In the earliest tests made, the sera of Lambs 17 and 18 contained sufficient antibody to protect against 1000 or more mouse-intra-

peritoneal LD$_{50}$ of pantropic Rift Valley fever virus By the time they were 11 weeks old the antibody titre in both animals had declined to the point where quantitative tests employing several dilutions of virus had to be made in order to determine whether antibody was present However, antibody sufficient to neutralize 10 LD$_{50}$ of virus was still present in the serum of the unweaned lamb

TABLE IX —*Selected Results from Serial Weekly Protection Tests on Lambs Born to an Immune Ewe*

Serum of Lamb No	Age (days)	Weaned (weeks)	Virus dilution, log	Fate of mice		Average survival time (days)	Titre of virus, 1 in—
				Died	Lived		
Normal control			5	6	0	1 3	
17	45	2	5	1	5	9 2	
18	45		5	0	6	10 0	
Immune control			5	0	6	10 0	
Normal control			4	6	0	1 3	30,200,000
			5	6	0	1 2	
			6	6	0	2 0	
			7	4	1	4 0	
			8	1	5	8 7	
			9	0	6	10 0	
17	129	14	4	6	0	2 0	3,170,000
			5	6	0	2 0	
			6	5	1	5 0	
			7	1	5	8 8	
			8	0	6	10 0	
18	129		4	5	1	3 2	317,000
			5	5	1	3 5	
			6	1	5	8 7	
			7	1	5	8 7	
			8	0	6	10 0	
Immune control			1	0	6	10 0	< 10
			2	0	6	10 0	
			3	0	6	10 0	
			4	0	6	10 0	
Normal control			5	6	0	1 7	56,000,000
			6	6	0	1 7	
			7	6	0	2 0	
			8	2	4	7 3	
			9	0	6	10 0	
17	150	17	5	6	0	2 0	3,170,000
			6	4	2	5 8	
			7	2	4	7 5	
			8	0	6	10 0	
18	150		5	5	1	3 2	10,000,000
			6	5	1	3 2	
			7	4	2	5 7	
			8	0	6	10 0	
17	157	18	5	6	0	2 0	10,000,000
			6	6	0	2 8	
			7	3	3	7 2	
			8	0	6	10 0	
18	157		5	6	0	1 7	20,500,000
			6	6	0	2 0	
			7	3	3	6 2	
			8	2	4	7 7	
Immune control			1	0	6	10 0	< 10
			2	0	6	10 0	
			3	0	6	10 0	
			4	0	6	10 0	

Horizontal rulings demarcate different tests with separate controls

143 days after its birth, and in the weaned lamb this amount of antibody was demonstrable on the 150th day

In the earliest studies made in this experiment there was evidence that the inherited immunity in Lambs 17 and 18 was less effective than in ovines convalescent from experimental infection, in that the neutralizing power of the sera of the former was less Nevertheless, the presence of antibody was found to be demonstrable in 2 ways by the capacity of the serum to prevent the lethal effects of the virus, and by the effect of the serum in prolonging the survival time in mice In fact, the average survival time proved to be an efficient indicator of the presence of antibody when the quantity of protective substance was inadequate to prevent death in a significant number of mice For example, in the 6th week after weaning, the serum of Lamb 17 protected only 1 of 6 mice from the lethal action of the virus, yet the average survival time of the group was 4 times that of controls receiving the same amount of virus mixed with non-immune serum Conversely, a progressively diminishing average survival time was an effective indicator of the decline of immunity The average survival time of mice receiving 1 in 100,000 suspension of infective liver tissue mixed with serum taken from Lamb 18 in the 7th, 10th, 13th, 16th, 19th and 22nd weeks of life was, respectively, 10 0, 4 3, 3 8, 3 3, 1 8 and 1 7 days

From the foregoing experiment it was evident that the inherited humoral immunity was lost very gradually, and that weaning had no effect on the rate of disappearance of the antibody from the serum

DISCUSSION

The spontaneous adaptation of Rift Valley fever virus to the nervous system upon serial intracerebral passage in mice, with concomitant diminution in its hepatotropic affinities, seems to indicate the presence of thinly-veiled neurotropic affinities in the native, unmodified virus These neurotropic affinities gain ascendency under circumstances which are favourable for their prompt action and which, at the same time, are not notably unfavourable for the hepatotropic affinities Whether the reversion of the neurotropic Rift Valley fever virus to the hepatotropic form can be brought about is not evident from these experiments, but as the adaptation to neural tissues is gradual and progressive, one might assume that reversion could take place up to a point, after which it would be progressively less likely Studies made by S F Kitchen indicate this to be the case

The neurotropic virus in our hands has never become completely innocuous for mice when introduced extraneurally, nor has it been determined whether the relatively small percentage of mice which succumb to extraneural inoculation do so from the effects of the virus on the liver or on the nervous system Nevertheless, the neurotropic adaptation of the agent results in a profound modification of its affinities for the tissues of lambs, in that the pantropic virus invariably enters the blood, whereas the neurotropic, in our experience, does not The evidence available furthermore indicates that adaptation of the virus to nervous tissues is associated with a decline in antigenic potency, as the pantropic virus seems to induce a higher titre of antibodies Nevertheless, sufficient antigenic potency is retained by the modified virus to stimulate the formation of antibody, which not only neutralizes the pantropic virus *in vitro*, but also vitiates its effects *in vivo*

2

Adequately comprehensive investigation of the value of neurotropic Rift Valley fever virus as a practical immunizing agent is beyond the scope of this laboratory at present   However, the results obtained to date give a hopeful outlook, and point to the desirability of further laboratory and field investigations The fact that the age of maximum susceptibility to Rift Valley fever in sheep falls in the earliest days of life (Daubney and Hudson, 1931) poses an administrative problem in the application of any preventive agent, and perhaps raises additional hazard on account of the tender age of the subjects   The observation that immunity to the disease in the ewe is transmitted to her offspring and persists for some weeks in the latter offers a hopeful lead   It is suggested that the inoculation of ewes with the neurotropic virus at, or before, the time of the mating season would induce immunity, which would be transmitted to the offspring, and might suffice to tide the latter over the age of maximum susceptibility Furthermore, the ewes, being immune, would not be subject to the hazard of abortion which is associated with natural infection with Rift Valley fever

SUMMARY

Several strains of Rift Valley fever virus which were passed in mice by serial intracerebral inoculation acquired enhanced neurotropism with concomitant reduction of hepatotropic affinities

Neurotropic adaptation of the virus could be brought about under controlled conditions by serial brain passage in mice   Parallel serial liver passage resulted in retention of the hepatotropic properties and no enhancement of neurotropism

The intraperitoneal inoculation of neurotropic Rift Valley fever virus into mice resulted in enhanced resistance to intracerebral inoculation of neurotropic virus and in complete immunity to pantropic virus

The subcutaneous inoculation of neurotropic Rift Valley fever virus into newborn lambs or older sheep resulted in the formation of specific antibody demonstrable by protection tests on their sera, and of immunity to the effects of inoculation with the pantropic virus   The neurotropic virus was not demonstrable in the circulating blood of lambs or sheep and caused no objective reactions of consequence

Humoral immunity inherited by twin lambs from their immune mother was demonstrable for about 5 months   Weaning of one of the twin lambs did not appreciably affect the rate of disappearance of the inherited immunity

Immunization against Rift Valley fever is discussed, and a suggestion is made for the practical application of a vaccine

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HYPERPLASIA OF THE PARATHYROIDS ASSOCIATED WITH OSTEITIS FIBROSA IN RATS TREATED WITH THIOURACIL AND RELATED COMPOUNDS

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SINCE the discovery of the goitrogenic action of thiourea, thiouracil and their derivatives (Richter and Clisby, 1942 , Kennedy, 1942 , MacKenzie and MacKenzie, 1943 , Astwood, Sullivan, Bissel and Tyslowitz, 1943), a vast literature has arisen describing the effects of such compounds on the thyroid, pituitary and other organs of the rat As far as we are aware, no report has been published describing changes in the parathyroids after the administration of these drugs It seems reasonable that such changes have been seen by other workers We ourselves have been familiar with their occurrence since our earlier studies on the goitrogenic action of brassica seeds (Griesbach, 1941), but only recently have long-term experiments provided us with material permitting an appreciation of the changes induced by goitrogenic agents in the parathyroids The purpose of this preliminary communication is to describe these changes, and the simultaneous occurrence of osteitis fibrosa in rats treated with thiourea, thiouracil or methylthiouracil for long periods

MATERIAL AND METHODS

The rats used belonged to two colonies of albino rats, substrains of Wistar rats, imported into this country 10 years ago No foreign stock had been added to these colonies Animals of both sexes, 6–8 weeks of age, were chosen They received, in their drinking water, either thiourea 0 25 per cent, or thiouracil 0 05 per cent, or methylthiouracil 0 05–0 025 per cent The diet given consisted of bran 30 per cent, pollard 25 per cent, bone meal 15 per cent, pea meal 15 per cent, maize meal 15 per cent Use was made also of material obtained in Sheffield from rats which had received 8 mg of allylthiourea daily These rats belonged either to another substrain of Wistar rats, or were piebald rats fed on a diet consisting of 3 parts skimmed milk powder and 7 parts dried bread, to which cod liver oil and cabbage were added once weekly Thyroid digest was prepared by T H Kennedy by peptic digestion of G P thyroid tablets The daily dose given by stomach-tube or by injection corresponded to 5 μg d-l-thyroxine After killing the animals the parathyroids were left in their connection with the thyroid gland and fixed in 10 per cent formol saline or acid Zenker's solution After fixation in formol saline the bones were decalcified with 5 per cent trichloracetic acid in Susa's fixative and embedded in paraffin The sections were stained with haematoxylin and eosin, van Gieson or Weigert's

stain   The pituitaries were fixed in sublimate-formalin, sectioned at 2 5μ, and stained according to Martins' modification of the Mallory method (1933)

RESULTS

Parathyroids

The parathyroids of animals treated for a long period with antithyroid drugs are frequently so large that their increased size is already apparent at naked-eye inspection   Their colour is a shiny white, with an occasional slightly yellow tinge   On some occasions the parathyroid is quite prominent, sticking out above the surface of the thyroid   The histological study reveals further changes Normally, at low magnification, the parathyroid appears in haematoxylin and eosin preparations as a dark blue circular or oval area which is sharply defined (Fig 1)   In our control rats the outer border of the parathyroid reaches the capsule of the thyroid and does not bulge out into the surrounding tissue   Only a thin capsule separates the parathyroid from the thyroid   There is little connective tissue inside the gland, and large blood vessels are rarely found besides the main artery   A small cell with a nucleus of elongated shape, rich in chromatin, predominates   The cytoplasm is scanty and basophilic (Fig 2)   The cell borders are not clearly defined in haematoxylin and eosin preparations   Dispersed among these elements are a few cells which have a larger pale cytoplasm and a round nucleus   In our controls dark oxyphilic and so-called water clear cells were not found   The hyperplastic glands show a different picture   Such glands bulge frequently into the loose areolar connective tissue surrounding thyroid and parathyroid (Fig 3)   Sometimes lobe-like projections are seen to extend into the neighbourhood, and the thyroid tissue surrounding the parathyroid may show compression   This is best seen in the animals with inactive thyroids   Here obviously the parathyroids have grown at a much higher rate than the thyroids, the growth of which had been arrested by continued administration of thyroxine in form of a thyroid digest   Fig 5 illustrates this phenomenon, showing an inactive thyroid which surrounds like a crescent the large parathyroid, the size of which is about equal to that of the thyroid   This picture also indicates the mode of enlargement of the parathyroid by apposition of lobules and by expansion   The capsule of the gland is thickened, and septa divide the parathyroid into several lobes   Blood vessels follow these septa,

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EXPLANATION OF PLATES

Fig 1 —Normal thyroid and parathyroid   Note ratio of parathyroid to thyroid size   (× 23)
Fig 2 —Normal parathyroid from Fig 1   (× 100)
Fig 3 —Hyperplastic parathyroid and hyperplastic thyroid containing adenomata   (× 23)
Fig 4 —Detail from Fig 3 showing pale oxyphil cells, alveolar arrangement   2 mitoses (× 450)
Fig 5 —Hyperplastic parathyroid in inactivated thyroid   Note ratio of parathyroid to thyroid size   (> 23)
Fig 6 —Cluster of dark oxyphil cells in hyperplastic parathyroid   (× 450)
Fig 7 —Transverse section of diaphysis of tibia, showing replacement of compact spongious bone   Same rat as in Fig 3 and 4   (× 15)
Fig 8 —Detail of a longitudinal section of tibia   Cellular fibrous marrow, replacing compact bone   Note osteoclast near trabecula   ×(100)
Fig 9 —Fibrous marrow containing spindle cells   (× 100)
Fig 10 —Osteitis fibrosa   Note numerous osteoclasts   (× 100)
Fig 11 —Osteoblasts lining trabecula   (× 450)
Fig 12 —Osteitis fibrosa with large haemorrhagic areas   (× 100)

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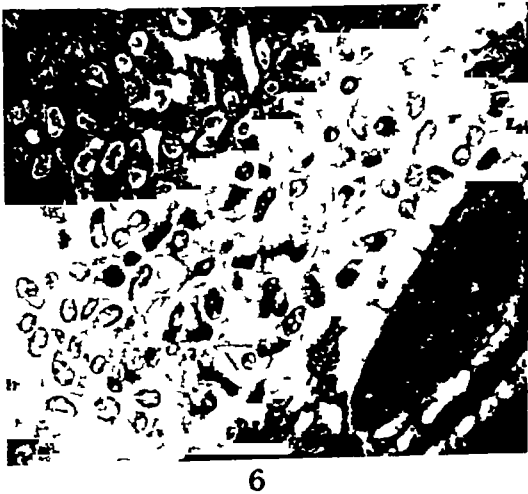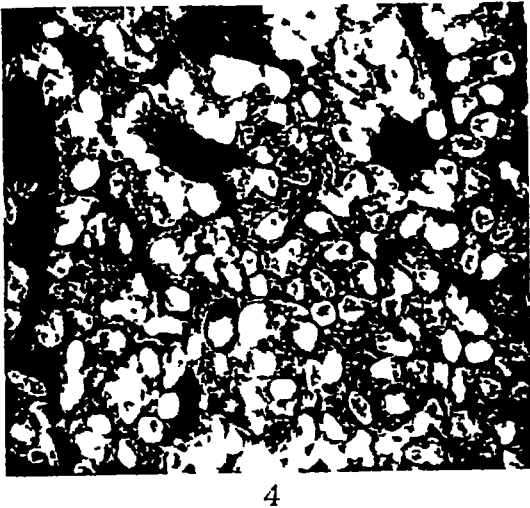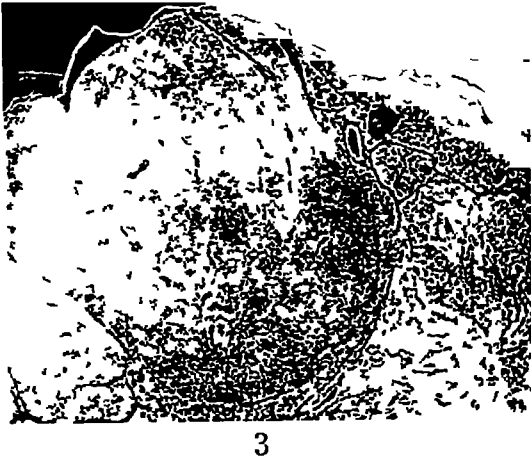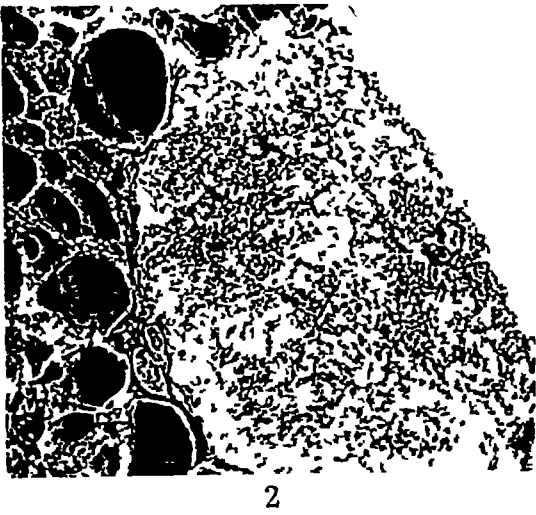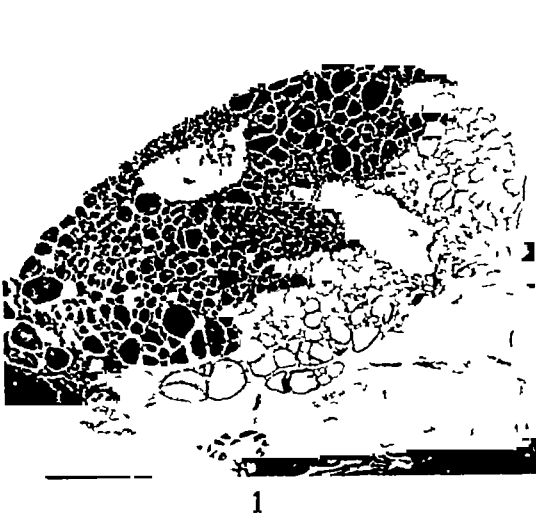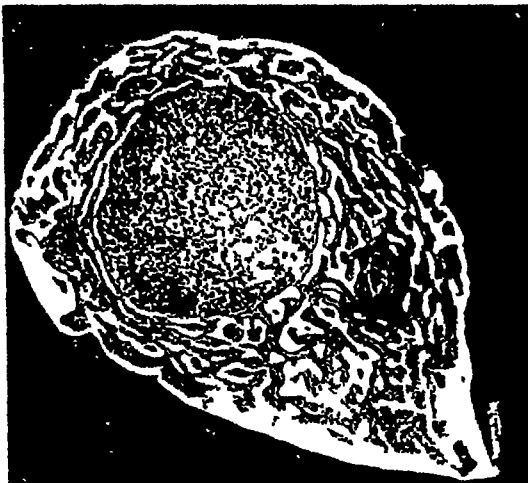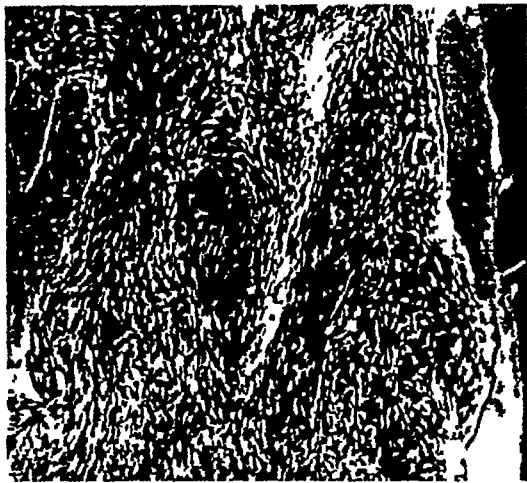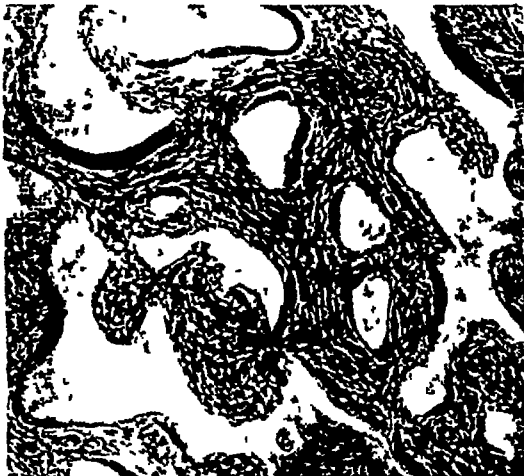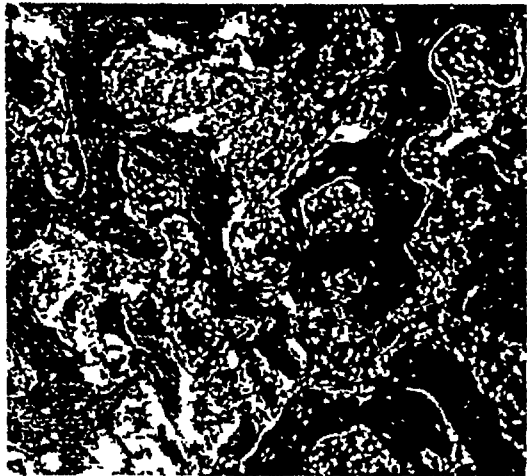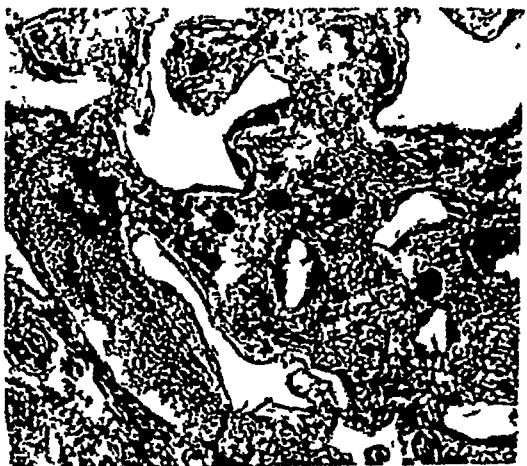
and frequently a general hyperaemia exists   In normal rats of our strain the longest diameter of the parathyroid in serial sections reaches 0 5–0 7 mm   In the hyperplastic glands diameters up to 2 2 mm have been found   More frequently were measurements of 1 2–1 6 mm

At low magnification the staining qualities of the enlarged parathyroids are generally lighter, and resemble more the colour of the surrounding hyperplastic thyroid   At first glance it is obvious that the cytoplasm of the predominating cell type is oxyphilic and the nuclei appear paler   At higher magnification one sees that the amount of cytoplasm is increased, staining a pinkish red, and that the cell borders are sharply defined   These cells are the well-known pale oxyphilic cells (Fig 4)   Their nuclei are round or oval with finely dispersed chromatin, which is displaced towards the quite conspicuous cell membrane   Frequently these cells form alveoli, which are surrounded by delicate strands of connective tissue   Such alveoli may show in the centre rests of pink or purplish colloid-like substance   Exceptionally large accumulations of this colloid were found   Clusters of dark oxyphilic cells with coarsely granulated cytoplasm appear in some of the largest glands (Fig 6)   Mitoses are frequent, especially in the periphery   Here three or more dividing cells can be seen in one field   It seems worth mentioning that in short-term experiments only one of the features described above was present regularly , besides a moderate general increase of the glands in size, there appeared to be an activation of the parathyroid cells   They were enlarged, and the nuclei were round and lighter in colour, due to the dispersal of chromatin described above   An occasional mitosis was found   In contrast to the experiments carried on for twelve months and more, this early hyperplasia did not occur when thyroid solution was given simultaneously with the goitrogen for 4 or 8 weeks and, consequently, the animals' thyroids were inactive   It seems logical to assume that the early parathyroid hyperplasia is due to the unspecific action of regional hyperaemia induced by the effect of the goitrogen

Bones

Once the fact of parathyroid hyperplasia induced by goitrogens was established, a systematic search for changes in the bones was made   Among the many animals studied, only a few were found showing a generalized disease of the skeleton (Table I)   All of these belong to the groups of rats which had received the goitrogen for considerable periods , bone changes have never been found in short-term experiments, i e 8–16 weeks   Fig 7–12 illustrate the essential features of the pathological process   A transverse section of the tibia (Fig 7) shows the normal fatty marrow with foci of haematopoiesis   The compact bone of the corticalis is replaced by spongy bone   Trabeculae of various size and appearance lie in a fibrous marrow which is sharply defined towards the fatty marrow   The connective tissue forms fibrous strands, which are fairly cellular in some areas (Fig 8), but show only a few spindle cells in others (Fig 9)   Weigert–van Gieson staining reveals the presence of delicate collagenous fibres   Adjacent to the trabeculae the number of cells present in the fibrous marrow increases, and two types of large cellular elements can be found   Many typical multinuclear osteoclasts are seen, frequently situated in Howard's lacunae (Fig 10)   Further, large osteoblasts surround other trabeculae (Fig 11), especially those which have a small rim of osteoid tissue   Engorged blood vessels

TABLE I

Treatment	No of rats	Rats with osteitis fibrosa	Sex of rats with osteitis fibrosa	Duration of experiment (weeks)
Thiourea 0 25%	14	1	M	71
		1	M	107
Methyl-thiouracil 0 01%	16	1	M	65
0 025%		1	F	71
0 05%		1	M	108
		1	M	122
Methyl-thiouracil 0 01% + 5 micrograms d-l-thyroxine daily (for 52 weeks)	3	2	M	56
		1	M	58

are frequently seen, blood extravasation or signs of old haemorrhages only rarely (Fig 12)   In the trabeculae fibrous bone instead of lamellar bone is prevalent Haversian systems are rare   The severity of the lesions varies greatly in different parts of the same bone   Sometimes one side of the diaphysis shows a normal corticalis, while only a rim of bone is left on the other side   In the most advanced cases the tibia had become so soft that it could be cut without previous decalcification   In the epiphysis essentially the same changes were found   Nearly all the fatty marrow had been replaced by fibrous tissue, and similar cell changes were present as in the diaphysis   As could be expected in these animals in which growth had stopped, the epiphyseal cartilage formed only a narrow zone   But when the epiphyses of normal rats of corresponding age were studied, it became apparent that the chondromedullary line (Gilmour, 1947) in rats with osteitis fibrosa showed obvious pathological changes   Whereas in the controls small columns of cartilage cells of fairly even size were present in the experimental animals, the fibrous marrow invaded the chondromedullary line   The cartilage cells nearest to the fibrous tissue were swollen, and seemed to undergo degenerative changes   At some points the activated fibrous tissue appeared to break through the zone of cartilage

The whole process is one of greatly increased resorption of bone which, as the study of less advanced lesions proves, starts in the Haversian canals   New bone is formed at the same time, but active apposition is exceeded by destruction   There is no sign of inhibition of calcification   Osteoid may be seen occasionally forming narrow seams round the calcified core of the trabeculae   However, there is no indication of disturbed intracartilaginous ossification as in rickets

Pituitary

The pituitary gland, after long-term treatment with thiouracil or methyl-thiouracil (0 025 and 0 05 per cent), presents uniformly the picture of degranulation of the chromophilic cell types   Therefore, only chromophobe elements

are present containing remnants of the basophil colloidal substance situated in intercellular spaces   Chromophobe cells, characterized as degranulated basophils by the typical situation of the negative image of the small Golgi body, are present in moderate numbers   But "thyroidectomy cells," abundant in the early stages of goitrogen action, are absent   The chromophobe cells generally do not contain enlarged Golgi bodies as after prolonged oestrogen treatment, and there is no evidence of actively secreting cells   Neither thiourea (0 25 per cent) nor methylthiouracil in weaker concentration (0 01 per cent) leads to complete degranulation of the acidophil cells   The pituitaries of the two animals which had received a maintenance dose of thyroxine together with the goitrogen for 12 months showed a cell distribution in the anterior lobe which corresponded to the normal picture   The only deviation worth mentioning was the more alveolar arrangement of the glandular cells round the blood capillaries, into which some basophils could be seen to secrete their contents   The pars intermedia in most of the old experimental animals was definitely enlarged   There was a higher number of cell rows made up by enlarged cells having round, well-staining nuclei   Numerous basophilic elements were distributed amongst the ordinary intermedia cells   An unusual number of cysts were visible, surrounded by the intermedia cells   These cysts appeared to be free of any staining material

DISCUSSION

Thyroid hyperplasia as produced by thiourea and related compounds is always accompanied by enlargement of the parathyroids   This could be observed as early as 3 weeks after the beginning of drug treatment   It seemed obvious that this effect was due to an increased blood supply to both the thyroid and the parathyroid   In fact, by simultaneous administration of the goitrogen and thyroxine, it could be shown that the hyperplasia of both organs could be suppressed in short-term (8 weeks) experiments   However, when goitrogen and thyroxine were given together for 12 months or more, a quite different picture resulted   Whereas the thyroids had retained their normal size and activity, the parathyroids increased in size proportionally to the time of treatment, reaching in some cases the size of the thyroid   Therefore the regional hyperaemia cannot be an essential factor in the genesis of parathyroid hyperplasia observed in the long-term experiments

The study of numerous animals has convinced us that hyperplasia of the parathyroid preceded by many months the appearance of bone lesions   These bone lesions are characterized by bone destruction, and also by a limited apposition of new bone   The syndrome observed corresponds closely to the picture of *osteitis fibrosa generalisata*, or Recklinghausen's disease of human pathology   This similarity is underlined by the importance of a time factor in both conditions   Only recently Alexander, Pemberton, Kepler and Broders (1944) have pointed out that not so much severity as the duration of hyperparathyroidism is responsible for the disease in humans

Our findings are highly suggestive for the assumption that the syndrome described is due to hyperparathyroidism, but we are not yet in a position to explain its ultimate cause   Possibly biochemical studies, now in progress, might shed further light on this problem   At the moment we can only state that the mode of action of the goitrogens on the thyroid must be altogether different from

the one on the parathyroid , thyroid function is regularly affected after a few days, while hyperparathyroidism develops only slowly, and in a small percentage of our animals   The literature on experimental hyperparathyroidism deals mostly with the effect of massive doses of parathormone given for short periods The changes in the bones in such experiments are similar to the ones described in this paper   However, the importance of the results obtained by Jaffe, Bodansky and Blau (1931) has been questioned by Snapper (1938) and Hunter and Turnbull (1931), because similar changes were found to occur in a great variety of experimental conditions and even spontaneously   Young animals had been used in the majority of such experiments, and in these, osteofibrosis seems easily induced   In our material we have not seen a single animal with osteofibrosis which was younger than 14 months

Apart from primary hyperparathyroidism (Recklinghausen's disease), enlargement of the parathyroids has been described in a number of conditions   All of these have in common a primary disturbance of the metabolism of calcium and phosphorus (Luce, 1923 , Campbell and Turner, 1942), as in rickets and osteomalacia, or they are due to acidosis as in renal failure (Gilmour, 1947) Only in the last condition do bone lesions occur, associated with enlarged parathyroids and resembling Recklinghausen's disease   Pappenheimer (1936) has studied this syndrome in young rats, the renal tissue of which had been greatly reduced   We have no evidence to assume that a kidney disease was the cause of the syndrome described in this paper   Further, it seems necessary to mention that the diets given in our experiments provided an ample supply of both calcium and phosphorus, excluding a nutritional deficiency

The most controversial issue in parathyroid physiology is the role of the pituitary in regulating the parathyroid function and size   Up to date no conclusive evidence for the existence of a parathyrotropic hormone has been obtained   We have studied the pituitaries of our rats suffering from hyperparathyroidism, and have failed to find any consistent changes in the cells of the anterior lobe   Our material reveals that parathyroid hyperplasia occurred independently of the presence or absence of acidophil cells   There were also no signs indicative for an active secretion of basophils or chromophobe cells We have however, observed an enlargement of the pars intermedia, accompanied by certain structural changes, the significance of which is not yet clear   As long ago as 1924 attention to an enlargement of the pars intermedia following parathyroidectomy was drawn by Vines (1924)

SUMMARY

(1) Long-term administration of goitrogens leads to an enlargement of the parathyroids in the rat

(2) After 14 months or more of feeding of thiourea or related compounds (thiouracil and methylthiouracil), a generalized osteitis fibrosa was found in 9 of 33 animals in association with enlargement of the parathyroids

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THYROTROPIC HORMONE IN THYROTOXICOSIS, MALIGNANT EXOPHTHALMOS AND MYXOEDEMA

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SINCE the discovery of the thyrotropic secretion of the pituitary by Aron, Loeb and Basset (1929), there has been much investigation of its function in controlling the thyroid gland in animals   In particular, it has been demonstrated that the hyperplasia produced by goitrogenic agents is produced by the thyrotropic secretion of the pituitary which can be detected in the blood (Griesbach and Purves, 1943)

However, there has been less success in evaluating the role of this hormone in human clinical syndromes   Hertz and Oastler (1936), using the hypophysectomized rat as a test animal, were unable to demonstrate thyrotropic activity in blood or urine of thyrotoxic patients, although positive results were obtained from myxoedema patients   Cope (1938) was also unable to find any increased thyrotropic hormone in the serum or urine of thyrotoxicosis patients He used the guinea-pig as a test animal, and showed that the method would detect thyrotropic hormone in the blood of the thyroidectomized rabbit Galli-Mainini (1942) assayed sera by their metabolism stimulating effects on thyroid tissue slices   He found no increased activity in sera from ordinary thyrotoxic patients, but obtained positive results in cases with dissociated ophthalmopathy

Nevertheless, it has been assumed by many writers that the syndrome of

thyrotoxicosis is primarily due to an excess of thyrotropic hormone production
It is also now the custom to ascribe the eye signs of the thyrotoxicosis syndrome
to the action of excessive amounts of the thyrotropic hormone on the orbital
tissues   There are a number of investigations made in animals which tend to
show that pituitary extracts having thyrotropic activity do have an effect on
the orbital tissues (Smelser, 1937 , Dobyns, 1945 , Albert, 1945)   The hypo-
thesis of over-secretion of thyrotropic hormone in thyrotoxicosis is, therefore,
an attractive one, since this single assumption would explain the two otherwise
unrelated features of the disease, namely, the excess of thyroid secretion and
the ocular signs   However, unless the thyrotropic hormone is found to be
present in the blood, it will be necessary to abandon or modify the hypothesis
From the results of our investigations it seems to us that the hypothesis must
in fact be abandoned

No entirely satisfactory method for the assay of human serum for thyrotropic
activity has been described   It is necessary to have a sensitive test animal,
and also one which can tolerate large injections of serum   We have not
considered the possibility of concentrating the hormone from the serum because
of the dangers of modifying the activity during the concentration process   Thus
Albert, Rawson, Merrill, Riddell and Lennon (1946) described an increase in
the activity of inactivated pituitary extracts after heating, either alone, or
with reducing substances   Therefore, despite the fact that the chick may be
the most sensitive test animal, we have chosen the guinea-pig as the test animal
because of its ability to absorb large doses of serum

In order that the smallest activation of the thyroid may be of significance,
it is necessary to treat the guinea-pigs so as to bring their thyroids as far as
possible into the completely inactive state   For this purpose we administered
thyroid substance which had been brought into solution by peptic digestion,
as has been previously described (Griesbach and Purves, 1943)   The response
of the thyroid to thyrotropic hormone was evaluated by measuring the mean
cell height of the acinar epithelium

MATERIAL AND METHODS

Litters of guinea-pigs, five to seven days old, were taken and injected with
thyroid digest, each daily injection containing 10 μg of iodine   After nine
days of thyroid digest treatment serum injections were begun   The serum
was given in the dosage of 2 5 ml per 100 g body weight per day, the guinea-pigs
being weighed each day for the determination of dosage   The daily dose was
given in two injections administered subcutaneously with several hours between
injections   In many cases the serum caused a local or general toxic effect, which
did not seem to be due to bacterial contamination, but at the dose level used the
animals usually survived the course   The serum was given on four successive
days   On the fifth day the animals were killed with coal gas and the thyroids
dissected out and fixed in 10 per cent formol saline   The sections were cut at
5u thickness and stained by Heidenhain s Azan method   For the measurement
of cell height the sections were projected on to an opaque screen at a magnifi-
cation of 1000   Cell heights in 50 acini were measured on the screen in mm ,
each mm representing one micron   Serum samples were separated from the
clot, cleared by centrifuging and preservatized with 0 3 per cent of metacresol

RESULTS

The sera examined comprised 22 sera from 19 cases of uncomplicated thyrotoxicosis before treatment, 19 sera from 17 cases of thyrotoxicosis under treatment with thiourea or thiouracil, 37 sera from 27 cases of malignant exophthalmos and 5 sera from 5 cases of myxoedema

Owing to a shortage of animals and deaths among them during the injections, there has not been a control obtained from every litter of guinea-pigs used for these experiments However, sufficient controls have been obtained to provide a continuous check on the unstimulated state of the guinea-pig thyroids The results obtained are summarized in Table I, and the distribution of the values is illustrated by the diagram of Fig 1
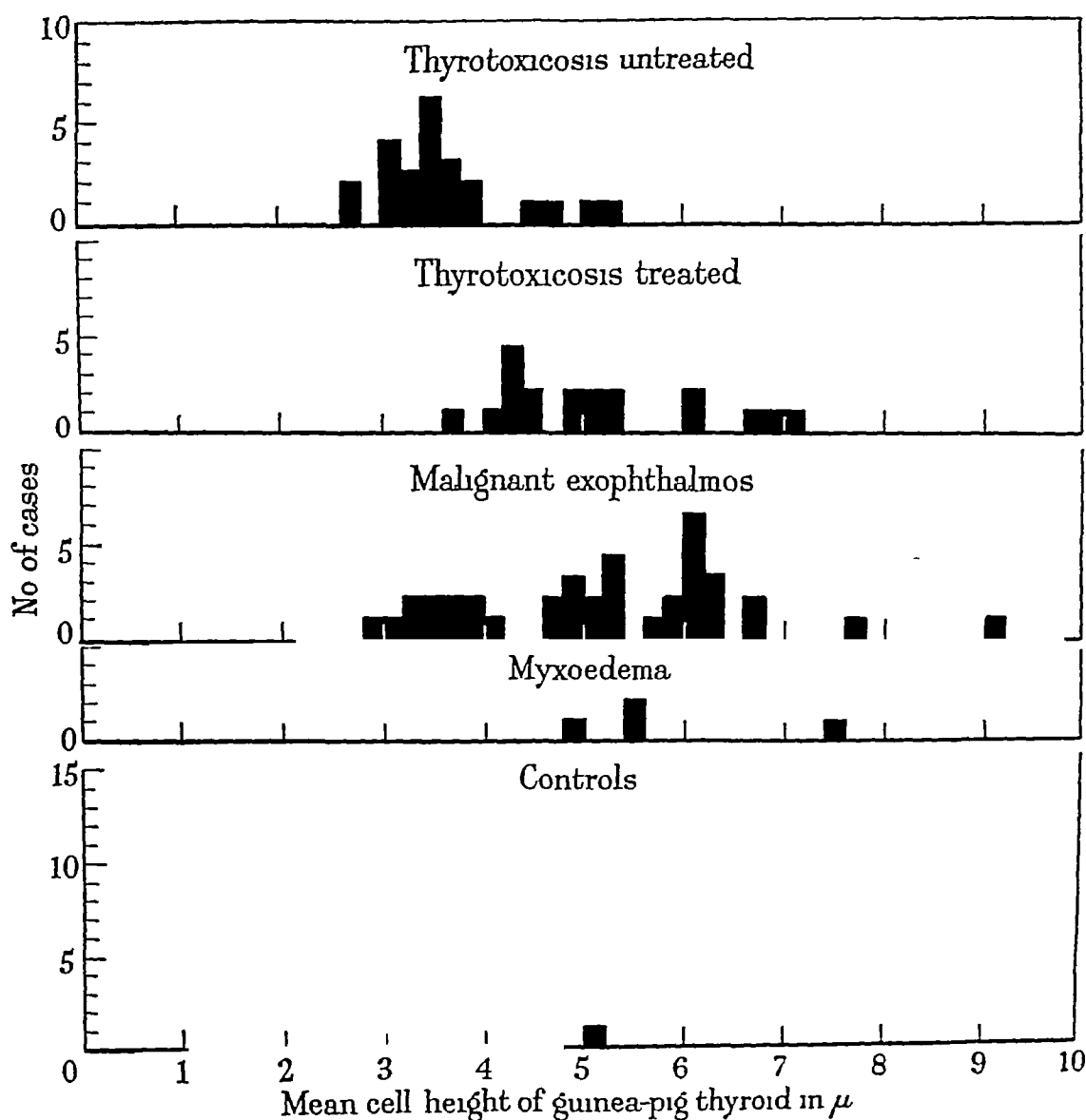
FIG 1 —Distribution of results of thyrotropin tests

TABLE I —*Results of Thyrotropin Tests on Human Sera   Mean Val*
Thyroid Cell Heights on Guinea-pigs

Sera	No of sera examined	Mean cell height	Standard
Control (no serum)	40	3 58	± 0
Thyrotoxicosis untreated	22	3 60	± 0
Thyrotoxicosis treated	19	5 09	± 0
Malignant Exophthalmos	37	5 21	± 0
Myxoedema . .	5	5 31	± 0

TABLE II

Comparison	Difference	Standar
Thyrotoxicosis untreated—controls	0 017	± 0
Thyrotoxicosis treated—controls	1 51	± 0
Malignant Exophthalmos—controls	1 62	± 0
Myxoedema—controls	1 73	± 0
Thyrotoxicosis treated—untreated	1 50	± 0

Comparisons that may usefully be made between these values ar
in Table II together with the standard errors of the differences   It wil
that there is no significant difference between the results of guinea-pigs i
serum from thyrotoxic patients and the control guinea-pigs   On tl
hand, the three groups, thyrotoxicosis under treatment with thioure
derivatives, malignant exophthalmos, and myxoedema, all show si
increases in cell height as compared with the controls   It is plain, tl
that thyrotropic activity was present in these sera   However, an exar
of the mean value does not present the whole story of the thyrotropic
of these cases   It is necessary to consider the amount of variation
groups   For instance, in the group "thyrotoxicosis untreated," the s
deviation of the cell heights was greater than that for the controls (σ fo
toxicosis untreated ± 0 70μ, for controls ± 0 47μ)   An examination
figures showed that this extra variation in the thyrotoxicosis group
to three abnormal values for cell height   These three values were 2 6,
5 3   With these three values eliminated, the standard deviation of the re
19 values was practically the same as that of the controls   The lov
height found in any of the control guinea-pigs was 2 9μ, and the highe
We have considered the possibility of the two low values in the "thyrot
untreated ' group being due to a thyroid depressing activity in these s
the evidence is insufficient to support this   The high value, 5 3μ, coulc
to the presence of thyrotropic activity in this serum, but the result i
high statistical significance, and could well be due to chance variation   Tl
in the thyrotoxicosis group with possible exception of one serum there
indication of thyrotropic activity

     The standard deviation for thyrotoxicosis treated (± 1 03μ) and for m
exophthalmos (± 1 47μ) are significantly greater than that for the
(± 0 47μ)   This is to be expected, since thyrotropic activity is pre

varying amounts in the different sera   The standard deviation of the malignant exophthalmos groups is considerably greater than that of the thyrotoxicosis treated group, but this difference is not of great statistical significance (P between 0 1 and 0 05)

An examination of the distribution of the values in the malignant exophthalmos group, as shown in Fig 1, reveals that while most of the sera tested had definite thyrotropic activity, there was a group of nine sera yielding cell heights between 2 8 and 3 9μ in which thyrotropic activity seems to be definitely absent   It is certain then that thyrotropic activity is not universally present in the blood of patients with malignant exophthalmos

An examination of the distribution of the values for thyrotoxicosis untreated and after treatment with thiourea or thiouracil as shown in Fig 1 reveals that the whole group of patients under treatment shows a shift to the right as compared with patients untreated   This indicates that the appearance of thyrotropic activity in thyrotoxic patients under treatment with thiourea or thiouracil is general

DISCUSSION

It is of great importance to know for comparative purposes the response to be expected from normal sera   Cope (1938) did not obtain with normal sera any histological activation of the guinea-pig thyroid using a method somewhat similar to our own   We have been able to observe only four specimens of normal human serum, but with these four no activation of the guinea-pig thyroid was detectable as compared with the controls   We have at present in progress a further investigation of the thyrotropic activity of normal sera, but for the purpose of the present investigation we will assume that the effects obtainable from normal sera are negligible when tested by our method

The results obtained from untreated cases of thyrotoxicosis seem to us to demonstrate conclusively that the hypersecretion and the hyperplastic activation of the thyroid in this disease is not the result of thyrotropic secretion from the pituitary   This result seems most important, since the current literature reveals that many investigators are assuming on insufficient grounds that the hyperthyroid syndrome is due to hypersecretion of pituitary thyrotropin   This hypothesis, which would make the hyperthyroid syndrome a disease primarily of the pituitary or of some mid-brain centre, has strong attractions, since it would seem to offer a hypothesis which could explain both the general activation of the thyroid and the ocular signs of this disease   However, it seems that the results of these tests show that this hypothesis, despite its attractions, must be abandoned   In saying this we are not overlooking the auxiliary hypothesis put forward by Rawson, Graham and Riddell (1943) that the thyrotropin is inactivated by the thyroid in the course of producing its stimulation   It has been postulated that there may be a hypersecretion of thyrotropin which is not detectable, since the thyrotropin is rapidly inactivated by the hyperplastic thyroid   We think that this inactivation could, in fact, account only for the absence of thyroid stimulating action in the blood of the thyroid veins   If the thyroid is being stimulated by the blood which is reaching it from the arteries, then that stimulating action should be demonstrable in blood drawn from the general circulation and should reveal itself in the guinea-pig   The inactivation of thyrotropic hormone by the thyroid may result in a lower level of thyrotropic

activity in the blood than would be attained after thyroidectomy, but it should still be detectable

The results of the test of sera from thyrotoxic patients after treatment with thiourea or thiouracil show that thyrotropic activity appears in the blood during treatment This thyrotropic activity in the blood of thyrotoxic-patients under treatment seems to us to show that pituitary function is substantially normal in hyperthyroidism Thus, while thyrotropic activity is absent while the thyroxine level of the blood is high, there is a secretion of thyrotropin when the thyroxine level has been reduced to normal or slight below normal levels However, it could be claimed that the appearance of thyrotropic activity during treatment with thiourea or its derivatives is due to the reactivation of inactivated thyrotropic hormone by the action of the thiourea (Albert, Rawson, Merrill, Lennon and Riddell, 1947) The observations of Wegelin (1938) on the histology of the pituitary in 20 cases of hyperthyroidism are most important in this connection and seem to us to be crucial He found the histology of the pituitary in hyperthyroidism to be substantially normal with no signs of hyperfunction Since secretion of thyrotropin is associated with hypertrophy and hyperplasia of basophil cells which are easily recognized, the absence of such changes in hyperthyroidism indicated that there is no excess secretion of thyrotropin in this disease

Although the thyrotropin hormone is not involved in the production of the hypersecretion of the thyroid and the eye signs of ordinary thyrotoxicosis, it could still be responsible for the severer orbital changes of malignant exophthalmos The hypothesis that excessive thyrotropin secretion has a causal role in the production of malignant exophthalmos obtains its main support from the demonstration of the exophthalmos-producing effects of pituitary extracts in laboratory animals There has been, however, little attempt to demonstrate the existence of this excessive thyrotropin in the blood of such patients Our own tests show that a major proportion of such malignant exophthalmos cases do, in fact, have high amounts of thyrotropin in the serum Nevertheless, the existence of typical cases of this syndrome with a complete absence of thyrotropic activity in the serum raises important doubts concerning the causal role of thyrotropin in this syndrome The group of cases without thyrotropic activity in the serum did not differ in any clinical feature from the group with elevated thyrotropic activity Early cases and very severe cases appeared in both groups Moreover, high levels of thyrotropic hormone were found in myxoedema patients and in thyrotoxicosis patients after thiourea or thiouracil treatment

The infrequent occurrence of malignant exophthalmos compared with the numbers of thyrotoxic patients being treated shows that in most individuals the presence of excess thyrotropic hormone in the blood does not lead to malignant exophthalmos Orbital changes are also certainly not a marked feature of myxoedema It therefore appears that a high level of thyrotropic hormone does not in general produce a malignant exophthalmos, nor is a high level invariably found in malignant exophthalmos patients It should be noted that in some of the malignant exophthalmos patients in which no thyrotropic hormone was found the serum was taken in the early stages of the disease when the symptoms were increasing rapidly Therefore, it cannot be said that the absence of thyrotropic hormone was due to the subsidence of the thyrotropic hormone

after a period during which it had been high and had produced irreversible changes in the orbit   The conclusion that we reach is that there is a considerable degree of correlation between malignant exophthalmos and high serum thyrotropin, but there is not apparently a direct causal relation between the two Either malignant exophthalmos must be divided into two or more diseases, in only one of which thyrotropic hormone is a causal agent, and even then requires the existence of some other circumstances to produce symptoms of the severity of malignant exophthalmos, or the thyrotropic hormone and the malignant exophthalmos are only indirectly related   In the latter case we would postulate that both the exophthalmos and the hypersecretion of thyrotropin are the results of some primary lesion, presumably in the central nervous system

When we began this investigation we were inclined to the view that malignant exophthalmos and thyrotoxicosis were two aspects of the same disease   In both syndromes hyperthyroidism and orbital changes may be observed   In malignant exophthalmos orbital symptoms overshadow the metabolism disturbance, which may indeed be absent   On this view the condition which is here called malignant exophthalmos has been termed Graves' disease with disassociated ophthalmopathy   We expected that the severe eye symptoms of malignant exophthalmos would be found to have the same cause as the less severe eye symptoms associated with typical Graves' disease   The fact that so many of the malignant exophthalmos patients had high thyrotropic activity in the blood which was never found in the typical hyperthyroidism makes it likely that the two diseases are distinct

SUMMARY

A method for the assay of thyrotropic hormone in serum from human subjects is described

The results of the examination of 22 specimens from cases of thyrotoxicosis before treatment, 19 specimens from cases of thyrotoxicosis under treatment, 37 specimens from cases of malignant exophthalmos, 5 specimens from cases of myxoedema are described

In thyrotoxicosis untreated no thyrotropic hormone was found

In treated thyrotoxicosis weak or moderate thyrotropic activity was present in all cases

Malignant exophthalmos cases fell into two groups   One group was devoid of thyrotropic hormone   In the other group definite thyrotropic activity was present, in some cases extreme activity being found

Marked thyrotropic activity was found in untreated myxoedema

It is concluded that thyrotoxicosis is not the result of excessive secretion of thyrotropic hormone

The appearance of thyrotropic activity in thyrotoxic patients after treatment is held to show that pituitary function is substantially normal in thyrotoxicosis

The inconsistency of appearance of thyrotropic hormone in malignant exophthalmos cases is considered to disprove the causal role of thyrotropic hormone in the production of malignant exophthalmos

It is considered that the excess thyrotropin secretion and the eye lesions of malignant exophthalmos are the result of a lesion in the brain-stem which affects the orbit and the pituitary by neural pathways

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THE EFFECT OF ACRIDINE COMPOUNDS ON MITOSIS AND OXYGEN CONSUMPTION IN THE ISOLATED BRAIN OF THE EMBRYONIC CHICK

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THE effect of mitotic poisons on the metabolism of isolated tissues is of importance both as a means of selecting those substances with a high specificity towards cells in division, and as a means of investigating their mode of action In this respect the effect on oxygen consumption has a special importance, because of the conclusion of Fisher and his colleagues (Fisher and Henry, 1944) that inhibition of cell division is associated with the inhibition of a fraction of the total cellular respiration concerned in this process

Recently a series of acridine compounds has been shown to inhibit mitosis in cultures of chick fibroblasts, and has been made available through the kindness of Dr J H Wilkinson   The effect of four of these compounds, both on mitosis and on respiration, has been tested on the isolated brain of the embryonic chick

MATERIAL AND METHODS

The tissue used was the mid-brain vesicle of the 6-day chick removed from the embryo with its mesodermal and ectodermal coverings   At this stage there are many dividing cells in the ependymal layer which can be readily examined by the aceto-lacmoid squash method of La Cour (Darlington and La Cour, 1942) Although accurate mitotic counts are not possible, about 5 per cent of the cells in the preparations were in some stage of mitosis

Oxygen consumption was measured by a modification of the Cartesian diver micromanometer previously described (O'Connor, 1948)   This technique was

designed to measure the oxygen consumption of *Daphnia*, and certain further modifications have been necessary The medium was a strongly buffered Tyrode solution of the following composition

Tyrode solution	100 ml
Na_2HPO_4 2 11 per cent	24 ml
KH_2PO_4 2 04 per cent	6 ml

the solution being isotonic and of pH 7 6   The amount of buffer was considered an advantage since, in the divers, the tissue was suspended in a small amount of fluid   When removed from the embryo the brain tissue was placed in this medium, and cut into fragments about 1 mm square   Divers filled with medium were placed beneath the surface of the medium, and four or five fragments introduced into the necks of the divers   The divers were then centrifuged to make the tissue enter the bulb   Excess medium was removed, leaving the tissue fragments suspended in 20–30 μl of fluid   Subsequent steps of filling the divers followed the technique already described, and included the introduction into the necks of the divers of a solution of the acridine compound to be tested   In most cases the solvent was the medium, but in certain cases limits of solubility made it necessary to use normal saline   In these cases the alteration of the saline composition of the bulb contents resulting from the addition of the acridine solution affected neither mitosis nor respiration   All experiments were performed at 37° C , and the divers were filled with oxygen

The normal rate of respiration was measured for 45 minutes and recorded as the change in the flotation pressure   The actual amount of oxygen consumed was not calculated, but the change in flotation pressure was 100–150 mm water, and corresponded to an oxygen uptake of approximately 1 μl   At the end of 45 minutes the acridine solution was added to the bulb contents, and any alteration in the rate of oxygen consumption measured   In order to allow for the mixing of the added acridine solution, the change in flotation pressure was recorded during the period 40–120 minutes after its addition, and the percentage alteration in oxygen consumption calculated   Control experiments were performed in which medium containing no acridine compound was added to the bulb contents from the neck of the divers   In twenty such experiments the oxygen consumption following this manoeuvre averaged 96 0 per cent of the normal, with a standard deviation of 5 08 per cent, so that an alteration of oxygen consumption of less than 10 per cent could not be considered significant

After two hours' exposure to the acridine compound the tissue was washed from the divers and examined by the aceto-lacmoid squash method   In control experiments mitotic figures showed no abnormality (Fig 1), but with effective concentrations of acridine compounds recognizable changes occurred   The slightest change observed consisted of swelling and irregularity of chromosomes, which at slightly higher concentrations became fused to a pycnotic mass (Fig 2), the course of events resembling that described by Dustin (1947)   Since it was easier to recognize the fusion to a pycnotic mass, the concentration of acridine compound producing this change was recorded as the "effective mitotic concentration "   Although this was a convenient method of comparing the acridine compounds investigated, it is desirable to point out that the results may not be comparable with methods using mitotic counts and where the compound is applied for longer periods of time

3

EXPERIMENTAL RESULTS

(a) *Effective mitotic concentration*

This concentration in the case of 5-amino-acridine hydrochloride, 5-amino-10-methylacridinium bromide, 5-amino-1-phenyl-10-methylacridinium bromide, and 5-amino-2-trifluoromethylacridine hydrochloride is recorded in Table I

TABLE I —*The Effect of Acridine Compounds on Mitosis and on Respiration in the Isolated Brain of the 6-day Chick Embryo*

Compound	Effective mitotic concentration mg /100 ml	Respiratory inhibition—		
		at effective mitotic concentration %	at 10× effective mitotic concentration %	at 16 mg / 100 ml %
5 amino 1 phenyl 10 methylacridinium bromide	1 8	0	0	0
5 amino-10 methylacridinium bromide	0 7	0	0	0
5 amino acridine hydrochloride	0 8	< 10	20	30
5 amino 2 trifluoromethylacridine hydrochloride	0 9	< 10	68	100

The results are averages from a series of experiments as described above, and from those where the effective mitotic concentration was separately tested In these cases fragments of the brain tissue were exposed to varying concentrations of the acridine compounds in small test-tubes filled with oxygen, and adequate oxygenation assured by reducing the amount of medium to a layer 2–3 mm in depth   The results in these two types of experiment did not differ   Most experiments involved decreasing concentrations by two-fold dilutions, and there was a subjective element in determining the effective mitotic concentration Hence it is necessary to suggest that, for two results to differ significantly, one should be twice the other   From Table I it is seen that 5-amino-1-phenyl-10-methylacridinium bromide is less effective than the other three compounds, which show no significant difference

(b) *Effect on respiration*

The same four compounds as in the previous section were tested   In each case 20–30 varying concentrations were used, and the relationship of concentration to inhibition of oxygen consumption plotted in Fig 3, where the concentration is expressed on a logarithmic scale   Certain relevant information is extracted from the figure and included in Table I   Thus it is seen that, at the effective mitotic concentration, the inhibition of respiration is small, none being demonstrated in the case of 5-amino-10-methylacridinium bromide and 5-amino-1-phenyl-10-methylacridinium bromide, while in the case of 5-amino-acridine hydrochloride and 5-amino-2-trifluoroacridine hydrochloride the decrease is less than 10 per cent, and thus not certainly significant   At ten times the effective mitotic concentration there is, however, a considerable difference, there being no demonstrable inhibition in the case of 5-amino-10-methylacridinium bromide and 5-amino-1-phenyl-10-methylacridinium bromide, while 5-amino-acridine hydrochloride and 5-amino-2-trifluoromethylacridine hydrochloride produce inhibitions of 20 per cent and 68 per cent respectively
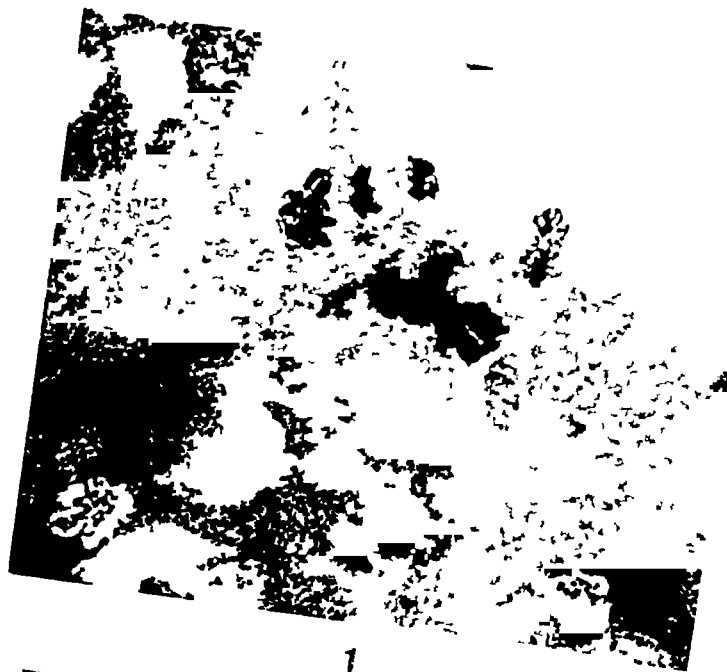
1
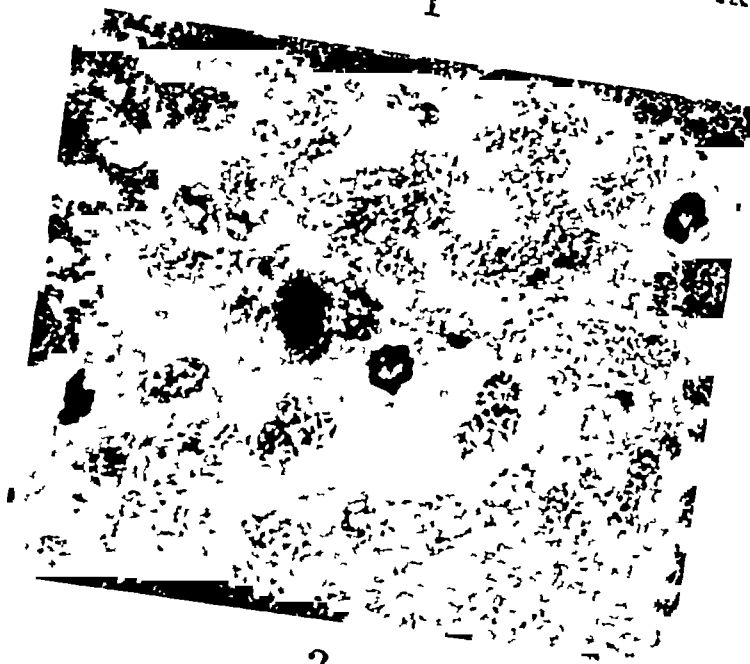
2

1 —Normal mitotic figures

2 —Mitotic figures after 2 hr exposure to an effective concentration of an acridine compound
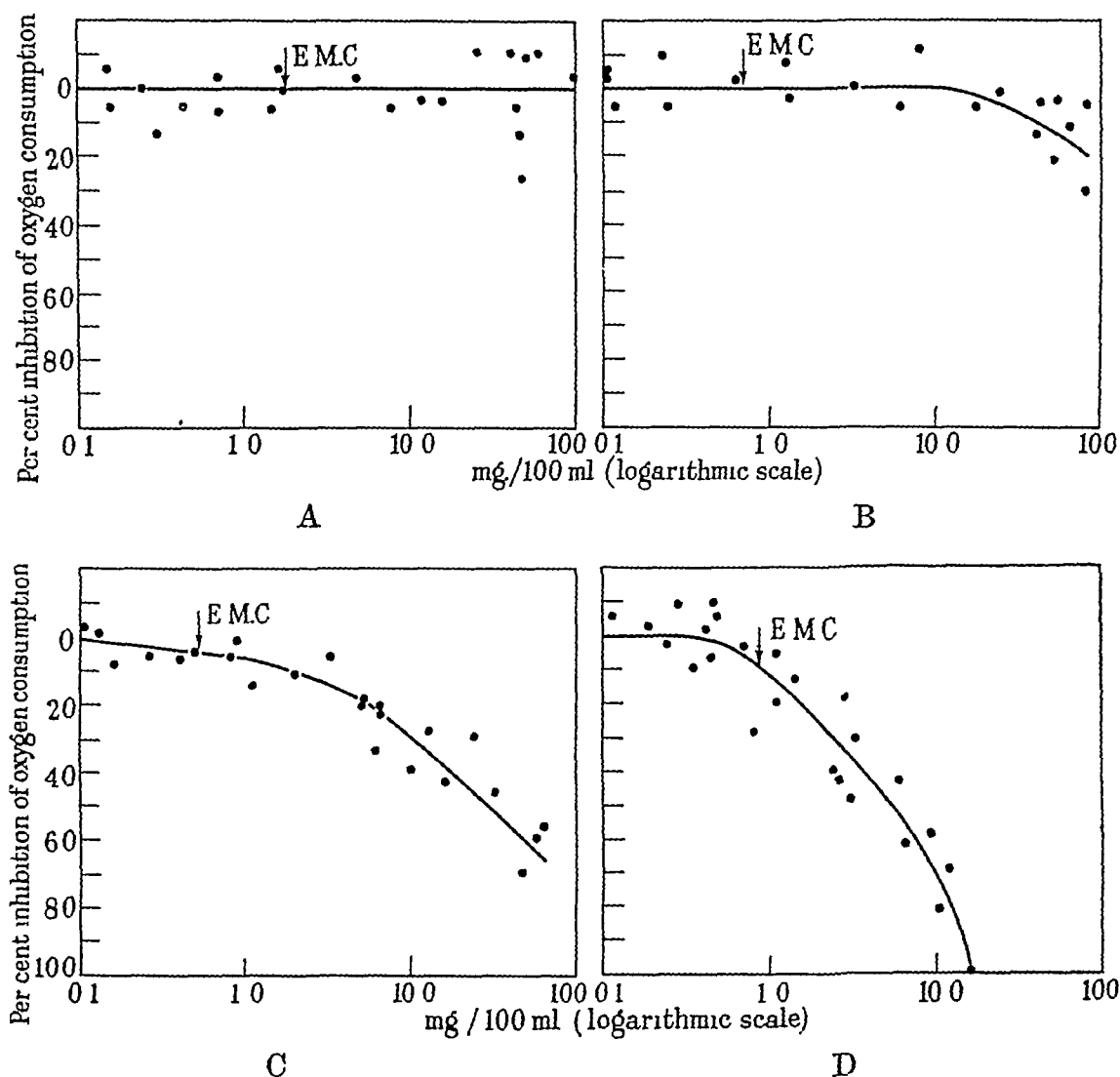
FIG 3 —The effect of acridine compounds on respiration   E M C = effective mitotic concentration
A   Concentration of 5 amino 1-phenyl 10 methylacridinium bromide
B   Concentration of 5 amino 10 methylacridinium bromide
C   Concentration of 5 amino acridine hydrochloride
D   Concentration of 5-amino 2 trifluoromethylacridine hydrochloride

DISCUSSION

Two of the compounds, 5-amino-acridine hydrochloride and 5-amino-2-trifluoromethylacridine hydrochloride, produce an increasing inhibition of respiration as the effective mitotic concentration is exceeded   Therefore, if these two compounds were used in intact animals, it is more likely that they would effect general cellular metabolism than the other two compounds

The inhibition of respiration cannot be due to effects confined to cells in division, since these comprise about 5 per cent of the total cells   It must therefore be due to an inhibition of the respiration of all cells   In the case of 5-amino-10-methylacridinium bromide, 5-amino-acridine hydrochloride, and 5-amino-2-trifluoromethylacridinium bromide hydrochloride, where the effective mitotic concentration is approximately equal, the inhibition of respiration, at equal concentrations, ranged from nil to 100 per cent (Table I)   It is therefore

concluded that the effect on mitosis and the effect on respiration are independent A comparable independence has been recorded with similar acridine compounds between antimalarial activity and the inhibition of the respiration of chick red cells infected with *Plasmodium gallinaceum* (Albert and Marshall, 1948)

It is of interest to compare these results with those of Fisher and his co-workers, who investigated the effect of urethane on yeast (Fisher and Stern, 1942), on *Tetrahymena* (Ormsbee and Fisher, 1944), and of both urethane and chloretone on the egg of *Arbacia* (Fisher and Henry, 1944) They concluded that total cell respiration could be divided into two fractions, one, the "active" respiration associated with cell division, and the other, the "resting" associated with other cellular processes They further concluded that the active respiration was more sensitive to the action of urethane and chloretone than the resting, and that inhibition of cell division was associated with the inhibition of the active respiration This, in different material, amounted to 20–40 per cent of the total respiration

In the present series of experiments with acridine compounds no similar decrease of respiration at the effective mitotic concentration has been demonstrated This difference may, however, be only apparent because of the lower rate of cell division in the material employed, so that any associated oxygen consumption might be so small that its inhibition could not be detected by the method used Therefore, it is not excluded that the action of acridine compounds on mitosis is associated with the inhibition of an associated fraction of the total oxygen consumption, which, when compared with the remainder, shows a greater susceptibility to the action of acridine compounds, and a smaller susceptibility to variations in their chemical structure

SUMMARY

(1) Simultaneous measurements have been made of the effect of four acridine compounds on mitosis and on respiration in isolated brain of the 6-day chick embryo

(2) All four compounds affected dividing cells in concentrations ranging from 0 7–1 8 mg per 100 ml , with little or no inhibition of respiration At higher and equal concentrations the inhibition of respiration ranged from *nil* to 100 per cent

(3) It is concluded that the effects on mitosis and on respiration are independent

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RIFT VALLEY FEVER, TRANSMISSION OF THE VIRUS BY MOSQUITOES

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A CONSIDERABLE amount of evidence was found by Daubney and Hudson (1931 1933) which indicated that the virus of Rift Valley fever is transmitted by mosquitoes   Nevertheless, it was not until 1944 that the agent was first isolated from wild-caught mosquitoes (Smithburn, Haddow and Gillett, 1948) and prior to the present communication there has been no account of its successful transmission by hematophagous arthropods

In 1944, while attempting to discover the vector responsible for the transmission of yellow fever among wild primates in uninhabited forest (Smithburn, Haddow and Gillett), Rift Valley fever virus was isolated 6 times from different lots of mosquitoes caught during a period of 39 days in a circumscribed area of the Semliki Forest in western Uganda   The agent was isolated once from *Aedes* (*Stegomyia*) *de-boeri* spp *de-meilloni*, Edwards, twice from the *Aedes* (*Aedimorphus*) *tarsalis*, Newst group, and 3 times in successive catches from *Eretmapodites* spp   The indications were that the former 2 contained little virus and may have been only casually infected, whereas the *Eretmapodites* not only were regularly infected during the period of virus activity, but apparently contained the virus in considerable quantity   These facts suggested that the local vector, if one was included in the species from which virus was isolated, was one of the *Eretmapodites* spp   Mosquitoes of the *Eretmapodites chrysogaster* Graham group were more numerous in the infected lots than were any of the other species of Eretmapodites   Moreover, members of this group were known to be capable of transmitting another virus disease—yellow fever (Bauer, 1928) Accordingly, it was decided to attempt the experimental transmission of Rift Valley fever virus with these mosquitoes, and with other species regarded as suspect vectors of the virus in Kenya   The tests with *Eretmapodites* were successful and are described in this paper, which therefore records the first experimental transmission of Rift Valley fever virus by the bites of mosquitoes

MATERIALS AND METHODS

At the time these experiments were carried out we had no colony of mosquitoes of the *E chrysogaster* Graham group, and we therefore were obliged to use wild insects or adults reared from wild-caught larvae   These were captured at Kitinda a forested locality near Entebbe, where they were present in good numbers and where Rift Valley fever was not known to have occurred   Adult female mosquitoes were caught individually in glass tubes as they alighted to bite the catchers, and the tubes were then lightly plugged with cotton   The captured insects were transported the short distance to the laboratory in the

catching tubes , there they were examined and sorted by one of us and released into Barraud cages

The *Taeniorhynchus* employed in the experiments were also wild mosquitoes, which were caught in lake-shore localities near this Institute   The *Aedes aegypti* were laboratory-reared insects descended through several generations from material collected in Nigeria by Dr J C Bugher, who supplied us with the mosquito eggs

The animals used were mice and lambs   The lambs were European-native hybrid stock and were about 5 or 6 months old   None of them died as result of Rift Valley fever in these or other experiments   Whether their survival was due to age, or breed, or to characteristics of the virus is not known   Their clinical and immunological responses to infection, whether induced by bites of mosquitoes or by inoculation, were typical

The mosquitoes received their infective feeds, in the first instance from mice sick or moribund following inoculation with virus of Rift Valley fever The inoculated mice were placed singly in close-fitting cylinders made of monel-metal gauze   Each end of the cylinder was closed with a cork to render the mouse relatively immobile without causing it discomfort   One of the corks was grooved to accommodate the animal's tail   In some instances moribund mice were exposed to mosquitoes without being confined in the cylinders   The infected mice in the cylinders (and the few not so confined) were placed in Barraud cages

The mosquitoes which gorged were removed to other Barraud cages   All those which fed on a given day on mice of the same group were placed in the same cage and given the same lot number   The cages were kept in a controlled temperature cabinet   The cages and the floor of the cabinet were covered with moist cotton in order to provide a highly humid atmosphere for the insects The temperature within the cabinet was 30° C throughout the experiments Mosquitoes of the genus *Taeniorhynchus* did not thrive well in the controlled temperature cabinet and, after a few preliminary trials, they were kept in large Barraud cages in the open air of the insectary, with only the tops of the cages covered with moist cotton   The temperature of the insectary varied between 22° C and 26 6° C during the experiments   The mosquitoes were given banana and water daily, but on the day before they were to be exposed to normal mice or a normal lamb for transmission attempts, the banana was removed from the cages

The mosquitoes to be offered a transmission feed on a lamb were placed individually in wide-mouthed gauze-covered glass tubes, which were applied to the shaven skin of the animal   The transmission feeds on mice were given in the same manner as the infective feeds

When the first transmission of the virus to a lamb was accomplished, fresh lots of mosquitoes were exposed to this animal   Those which gorged were thereafter handled in the same way as mosquitoes which received their infective feeds from mice

An animal which served as source of virus for the mosquitoes was usually bled from the heart in the case of a mouse (for this and other procedures the animals were anaesthesized with ether), or the jugular vein in the case of a lamb, immediately after the infective feed, and the serum was tested for virus   In the case of the lamb sera   titrations of virus content were done

During the experiments infected mosquitoes were inspected daily, in the early part of the morning Any which were found dead were ground up, usually individually, for inoculation into mice to determine whether they had retained the virus For these tests, mosquitoes were triturated in a mortar with 1 ml of 10 per cent non-immune serum in physiological saline (hereafter referred to as serum-saline or diluent) The suspension was spun in an angle centrifuge at about 2500 r p m for 15 to 30 minutes A group of mice was inoculated intraperitoneally, with 0 06 ml of the supernate per mouse, after which the remainder of the supernate was passed through a Seitz E K pad previously washed with serum-saline A second group of mice was inoculated intraperitoneally with this Seitz filtrate

When a lamb bitten by infected mosquitoes exhibited an elevation of temperature it was bled, and its serum was tested for circulating virus When quantitative virus determinations were required, end-points were calculated by the method of Reed and Muench (1938), and the end-point dilution was regarded as containing 1 LD_{50} per unit of inoculum

The livers of mice which sickened or died as the result of being bitten by infected mosquitoes were employed for passage to other groups of mice Inoculated mice were examined once daily, or oftener when necessary Protection tests were made to identify virus recovered from mosquitoes The identity of virus recovered from mosquitoes was further confirmed by histological examination of livers of mice inoculated with suspensions of the mosquitoes

The protection tests were done in the following manner 0 25 ml portions of material suspected of containing Rift Valley fever virus (lamb serum, or the unfiltered supernate of a 10 per cent suspension of liver from a sick or a dead mouse) were added to 0 5 ml portions of known non-immune serum and to specific Rift Valley fever immune serum After vigorous agitation the serum-virus mixtures were allowed to stand for 15 minutes to 1 hour, at either room or incubator temperature, after which separate groups of 6 mice were inoculated intraperitoneally with 0 06 ml of the mixtures per mouse The presence of Rift Valley fever virus was considered proved if not more than 1 mouse receiving the mixture containing non-immune serum survived and if all, or all but one, of those receiving the mixture containing Rift Valley fever immune serum remained well during a period of 10 days following inoculation Tests for immunity were made in convalescent lambs by mixing stock virus with the serum of the lambs in question This was incubated and inoculated in the same manner as the serum-virus mixtures for protection tests Suitable controls were employed

The macroscopic appearance of the livers of infected mice was found to be so variable as to be unreliable for diagnostic purposes, but the presence of Rift Valley fever could easily be detected by microscopic examination of this tissue In no instance was there disagreement between the results of protection tests and the histological examination

EXPERIMENTAL

Eretmapodites chrysogaster Graham group

Four lots of mosquitoes of the *E chrysogaster* Graham group were found to, or appeared to, have received their infective feeds at times when the level of circulating virus was adequate in the infecting animal and 3 of these lots

gave successful transmissions as will be seen from the following paragraphs
Table I shows the number of mosquitoes of each lot which bit normal animals
on various days and indicates those bitings which resulted in transmissions

TABLE I —*Mosquitoes of the Better Infected Lots of each Species, showing Number*
which Probed or Gorged on Normal Animals at Various Intervals after their
Infective Feeds

Days after infective feeds	No mosquitoes which probed or gorged on mice							No mosquitoes which probed or gorged on lambs						
	Aedes aegypti	Eretmapodites chrysogaster group				Taeniorhynchus fuscopennatus		Aedes aegypti	Eretmapodites chrysogaster group				Taeniorhynchus fuscopennatus	
	Lot 5	Lot 1	Lot 3	Lot 7	Lot 11	Lot 15	Lot 17	Lot 5	Lot 1	Lot 3	Lot 7	Lot 11	Lot 15	Lot 17
3					13									
5	4				Many									
6			3				2							
7	7				11									
8							Several					.		
9					Several								.	
10		2					4	7			4		.	
11			1		5				2					
12	1					2	3							
13					4		2			1	4†			.
14								5					.	
15					3								.	.
16													1	
17											7	.	.	1
18								5				.		
19					2*									
20				4*					3*		3*		1	10
21								1				.		
22					1						2			6
23													1	
24		1						2						4
25					1						1	1		
27													1	5
29											1			3
31											1			1
32													1	

* Transmission occurred
† Mosquitoes of Lots 1 and 7, which received their infective feeds 6 days apart bit Lamb 3
on the same day   It is probable that the transmission was effected by Lot 1

Transmission 1 —Twelve *E chrysogaster* group mosquitoes comprising
Lot 1, received their infective feeds from mice on May 12   Although they were
afterward repeatedly offered feedings on normal mice in attempts to secure
transmission of the virus none of them bit these animals   Two of the lot bit
normal Lamb 3 on the 11th day after their infection without results   Three
of the lot bit the same animal on the 20th day after their infection (June 1)
together with 4 *E chrysogaster* group mosquitoes of Lot 7 which had received
their infective feeds on May 18 6 days after the mosquitoes of Lot 1 (Table I)
On June 4 the lamb s temperature rose sharply to 107 2° F (Fig 1) and
remained elevated throughout the day but it was within normal limits by the
following morning
    Six tests for virus were made with mosquitoes of Lot 1 from the 15th to the
26th day after their infective feeds   Five of the tests were made with individual
mosquitoes and the 6th with a pool of 2   The pool of 2 mosquitoes and 3 of
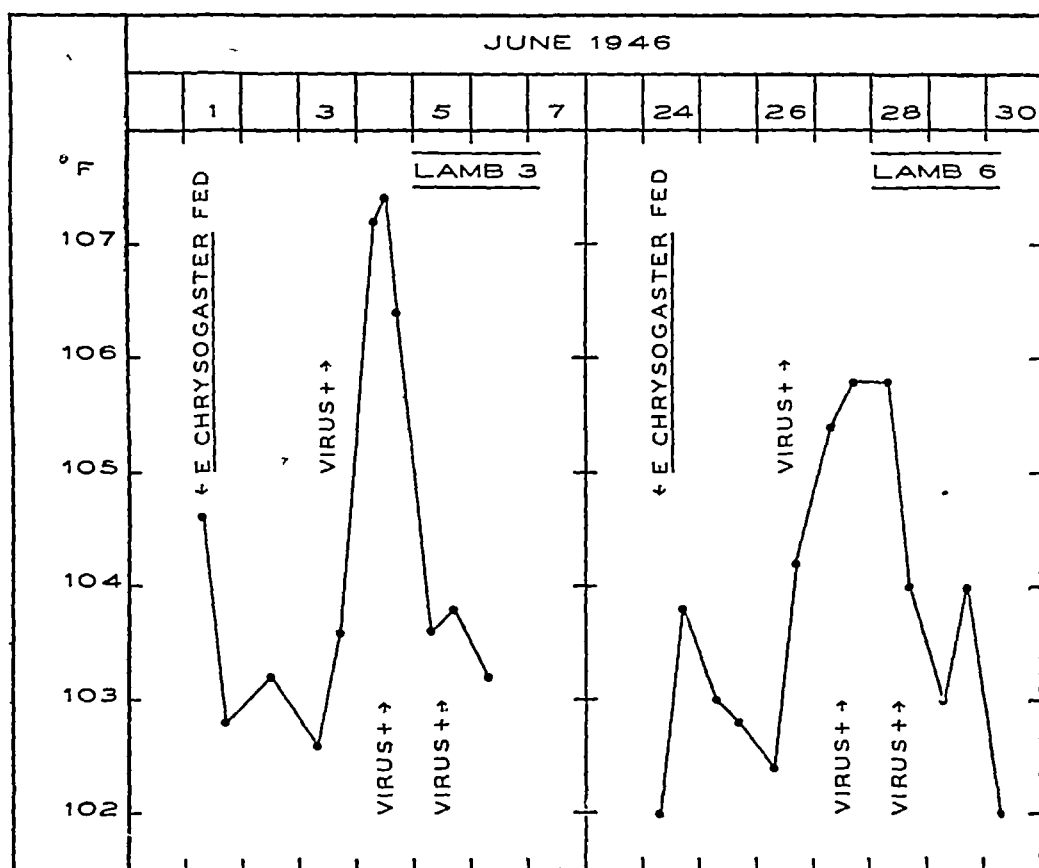
Fig 1 —Temperature charts of Lamb 3 and Lamb 6

the individual mosquitoes contained virus, so that either 4 or 5 of the 7 mosquitoes tested were shown to have retained the virus, all of them for more than 2 weeks

Lamb 3 was bled daily for 10 days, and groups of mice were inoculated intraperitoneally with its serum to test this for the presence of circulating virus Serum specimens taken on June 4 (the day of fever) and on June 5 were titrated, while on other days only the undiluted serum was tested   Sufficient virus was present in serum taken June 3 to cause the death of all the inoculated mice On June 4 the titre of virus in the serum of the lamb was 1 in 34,000, and on June 5 it was 1 in 69 (Table II)   No virus was present in blood taken on other days

A protection test employing as virus the serum taken from Lamb 3 on June 4 against normal and Rift Valley fever immune sera, showed that the infective agent present that day in the serum of the lamb was specifically neutralized by the Rift Valley fever immune serum (Table II)   Additional protection tests, employing preinfection serum, and specimens taken at various stages in the convalescence of the lamb, against stock Rift Valley fever virus, showed that demonstrable protective antibody was present 7 days after the onset of fever or 10 days after the animal was bitten by infected mosquitoes (Table III)

Histological studies were made of the liver of a mouse inoculated with serum taken from Lamb 3 on June 3   These revealed the presence of the characteristic lesions of Rift Valley fever, in further confirmation of the transmission

TABLE II —*Results of the Intraperitoneal Titration of the June 4 Serum of Lamb 3, and of a Protection Test which showed that the Pathogenic Agent in that Serum was Rift Valley Fever Virus*

Serum	June 4 serum, Lamb 3, dilution, log	Deaths of mice (days after inoculation)						Summary of result		Titre of virus, 1 in—
								Died	Lived	
Normal rhesus	0	2,	2,	2,	3,	3,	3	6	0	
,, ,,	2	3,	3,	3,	3,	3,	5	6	0	
Immune human	0	S,	S,	S,	S,	S,	S	0	6	
,, ,,	2	S,	S,	S,	S,	S,	S	0	6	
Diluent only, for titration of virus in serum of Lamb 3	0	2,	2,	2,	3,	3,	3	6	0	
	1	3,	3,	3,	3,	3,	4	6	0	
	2	2,	3,	3,	3,	3,	3	6	0	34 000
	3	3	3,	3,	3,	3,	S	5	1	
	4	3	3,	3,	3,	3,	S	5	1	
	5	3,	3,	S,	S,	S,	S	2	4	
	6	S	S,	S,	S,	S,	S	0	6	

S indicates that the mouse survived

It could not be determined whether Lamb 3 was infected by mosquitoes of Lot 1 or Lot 7   However, in the light of results which follow, it seems probable that it was infected by the mosquitoes of Lot 1, and that the extrinsic incubation period was 20 days

TABLE III —*Results of Two Intraperitoneal Mouse Protection Tests employing 1 per cent Rift Valley Fever Virus, Showing the Acquisition of Humoral Immunity by Lambs Infected through the Bites of* Eretmapodites chrysogaster *Group*

Serum		Days after infecting bites	Deaths of mice (days after inoculation)						Summary of result	
									Died	Lived
Lamb 3	preinfection		2,	2,	2,	2,	3,	4	6	0
	June 7	6	3,	3,	3,	3	3,	3	6	0
	,, 8	7	3,	3,	3,	3,	3,	3	6	0
	9	8	3,	3,	3,	3	4,	4	6	0
	,, 10	9	5,	5,	6,	6,	S,	S	4	2
	11	10	4,	S,	S,	S,	S,	S	1	5
	17	16	S,	S,	S,	S,	S,	S	0	6
Lamb 4	normal		2,	2,	2,	2	3,	3	6	0
5			2,	2,	2,	2	3,	3	6	0
1	immune		S,	S,	S,	S	S,	S	0	6
Lamb 4	normal		2,	2,	2,	2,	2	2	6	0
5	,,		2,	2,	2,	2,	2,	5	6	0
6	preinfection		2,	2,	3,	3,	3,	3	6	0
6	July 5	11	S,	S,	S,	S	S,	S	0	6
1	immune		S,	S,	S,	S,	S	S	0	6

S indicates that the mouse survived

Transmission 2 —The *33 E chrysogaster* mosquitoes comprising Lot 7 received infective feeds from mice on May 18   One gorged on and 2 probed a normal mouse on the 6th day after the infective meal, and another probed a normal mouse on the 11th day, but no transmission occurred   Four of the mosquitoes gorged on a normal lamb on the 10th day, without transmitting virus, another 4 bit a normal lamb (No 3) on the 14th day, probably with negative result   The final exposure to normal animals occurred on the 20th

day (June 7), when 4 of the insects bit a normal mouse (Table I) The mouse remained well during 48 hours, but was found dead on the morning of the 3rd day It was inadvertently discarded before a protection test was made or the liver was obtained for histological examination However, the death of the animal within the appropriate period of time and after an illness of less than 24 hours is indicative of Rift Valley fever Moreover, all the mosquitoes that bit this mouse were shown to be infected On the 21st day after the infective feeds the 13 mosquitoes remaining alive, including the 4 which bit a normal mouse the previous day, were tested individually for the presence of virus Each of the 13 contained virus Sixteen other tests for virus were made on individual mosquitoes of this lot between the 8th and the 20th day after the infective meals , 14 gave positive results The tests on this lot, therefore, showed that 27 of 29 mosquitoes tested had retained the virus, 13 of them for as long as 21 days The extrinsic period of incubation in this transmission was 20 days, and the intrinsic period was 3 days

*Transmissions 3 and 4 —*The 44 *E chrysogaster* group mosquitoes of Lot 11 received their infective feeds on Lamb 3 on June 4, at a time when the serum of the lamb contained 567,800 LD_{50} of virus per ml The lamb itself had been infected by *E chrysogaster* group mosquitoes Mosquitoes of Lot 11 bit normal mice on the 3rd, 5th, 7th, 9th, 11th, 13th and 15th days after their infective meals without transmitting virus A normal mouse bitten by 2 of the mosquitoes on the 19th day (June 23) became infected (transmission 3) Individual mosquitoes probed normal mice on the 22nd and 25th days, but got no visible blood, and did not transmit virus Five gorged on and 2 probed non-immune Lamb 4 on the 17th day, but transmission did not occur One gorged on and 2 probed normal Lamb 6 (Table I) on the 20th day (June 24), and this lamb became infected (transmission 4) Two mosquitoes probed normal Lamb 4 on the 22nd day and one on the 25th day, but got no blood and did not infect that lamb One mosquito gorged on normal Lamb 4 on the 29th day and again on the same lamb on the 31st day without result This mosquito was tested on the 31st day and found to contain virus Its failure to transmit is unexplained, but probably indicates that the vector potential of mosquitoes of the *E chrysogaster* group is variable Forty-two tests of individual mosquitoes of this lot showed that 33 of them retained virus

In summary, it may be said that transmission from lamb to lamb and from lamb to mouse was effected with mosquito Lot 11, with extrinsic incubation periods of 20 and 19 days respectively, and that 78 per cent of the mosquitoes tested were proved by inoculation to have retained the virus, the longest period of retention being 31 days

The normal mouse bitten by mosquitoes of this lot on June 23 (transmission 3) remained well during 4 days, but was sick on the morning of the 5th day It was sacrificed, and a suspension of a portion of its liver was used as virus in a Rift Valley fever protection test Of the 6 mice which received this suspension mixed with non-immune serum, 4 were dead on the 2nd and 2 on the 3rd day Each of the 6 mice receiving the liver suspension mixed with Rift Valley fever immune serum remained well during 10 days The result showed that the liver of the mouse contained an infective agent which was specifically neutralized by antibody against Rift Valley fever virus The transmission was further confirmed by the fact that the liver of the mouse infected by the mosquitoes of

Group 11 exhibited specific lesions of Rift Valley fever   The incubation periods in this transmission were   extrinsic, 19 days , intrinsic, 5 days

Lamb 6, bitten by mosquitoes of Lot 11 on June 24 (Transmission 4), remained afebrile and appeared well until the afternoon of June 26, when its temperature was slightly elevated and it seemed listless   The temperature was further elevated the next day (Fig 1), and did not return to normal until June 29th Tests in mice of serum taken from Lamb 6 showed that virus was present in its blood on June 26, 27, and 28, but not on other days   Quantitative tests were done on June 27 and 28, and titres of virus in the serum were 1 in 15,800 and 1 in 372 respectively   The liver of a mouse ill as result of inoculation with the serum of Lamb 6 taken on June 26 was used (as virus) in a Rift Valley fever protection test   Five of 6 mice receiving the suspension of this liver mixed with normal serum succumbed on the 2nd and 3rd days, while 5 of 6 receiving the liver suspension mixed with Rift Valley fever immune serum remained well , one mouse receiving the normal serum mixture survived, and one receiving the immune serum mixture died   This test showed that the infective agent in the serum of Lamb 6 was Rift Valley fever virus

Further proof of the transmission was the observation that Lamb 6 developed protective antibody against Rift Valley fever virus as a result of the experiment (Table III)   The incubation periods in this instance were   extrinsic, 20 days , intrinsic, 3 days

Lots of E chrysogaster *with which no transmission was effected* —Thirteen *E chrysogaster* group mosquitoes, Lot 3, received infective feeds from mice   Two of them (Table I) probed a normal mouse on the 10th day but got no blood , another bit a normal mouse on the 24th day, and one bit a lamb on the 13th day No transmission occurred   Ten of the mosquitoes were tested individually for virus 13 to 25 days after their infective feeds   Six of these were positive, 4 on the 25th day out of 6 tested that day   In the light of other findings, the only opportunity for transmission with this lot was in the case of the mouse bitten on the 24th day, and we cannot be certain that the mosquito which bit that mouse contained virus

Three other lots of *E chrysogaster* group were included in the experiments All the mosquitoes in 2 of the lots were dead by the 15th day, none having bitten normal animals after the 5th day , none of the third lot bit normal animals after the 18th day   No transmissions occurred   The 30 mosquitoes of the 3rd lot (Lot 12) received their infective feeds from Lamb 3, but after the peak of circulating virus in the animal was past, and when the titre of virus in its serum was 1 in 69, representing only 1152 effective (for mice) units of virus per ml of serum Each of the 30 insects in this lot was tested individually for virus   Only 6 of the 30 tests were positive, in sharp contrast with the results obtained with Lot 11, which fed on the same lamb at a time when the serum of the animal contained 567 800 LD_{50} of virus per ml of serum, and in which 33 of 42 mosquitoes tested were found to have retained the virus   Thus the causes of failures in transmission were probably the following   (1) failure of the insects to bite normal animals after an appropriate extrinsic incubation period, and (2) low levels of virus in the serum of the animals which were used to infect the mosquitoes

Table 4 shows the consolidated data on all the lots of the *E chrysogaster* group, and of other species of mosquitoes tested, and includes the results of tests for virus by inoculation as well as results of the successful and unsuccessful

TABLE IV —*Summary of Tests for Virus by Inoculation, and of Attempts to Transmit Rift Valley Fever Virus with 4 Species of Mosquitoes*

Species of mosquitoes		Aëdes aegypti	Eretmapodites chrysogaster group	Taeniorhynchus fuscopennatus	Taeniorhynchus uniformis
Number of lots		4	7	12	3
Number of mosquitoes		58	177	644	79
Tests for virus by inoculation into mice	Number*	49(49)	121(122)	263(348)	15(15)
	Positive	6	78†	40	6
Latest test for virus, day		37	37	38	14
Latest positive test for virus, day		26	31	29	13
Survival after infective feed, days	Minimum	1	1	1	1
	Maximum	36	36	37	13
	Mean	15 2‡	14 4§	7 4	5 2
Mosquitoes alive 20th day	Number	25	52	34	0
	Per cent	43 1	29 4	5 3	0
Last biting, day		34	31	34	0
Number of mosquitoes biting after 19th day	All lots	63	18	77	0
	Better lots	3	18	34	0
Successful transmissions		0	4	0	0
Incubation period, days	Extrinsic		19,20		
	Intrinsic		3–5		
Lots failing to show virus‖		2/32	0/–	3/75	2 lots not tested

* Figures in parentheses are numbers of mosquitoes included in the tests
† 24 of 30 in one lot were negative   Excluding these, the number retaining virus was 72 out of 91   The one poor lot was infected on a lamb after the peak of circulating virus was passed
‡ 7 mosquitoes were sacrificed on the 29th day, thus reducing the mean
§ 19 mosquitoes were sacrificed 20 and 24 days after the infective feeds, thus reducing the mean
‖ Numerator shows the number of lots, the denominator the number of mosquitoes tested in these lots

attempts to transmit by bite   It will be noted that the *E chrysogaster* group mosquitoes retain the virus much better than other species, and that they alone were successful in transmitting it

Experiments with other species

Transmission experiments with 4 lots of *Aëdes aegypti*, 12 lots of *Taeniorhynchus fuscopennatus*, and 3 lots of *Taeniorhynchus uniformis* which had taken blood from infected mice, were unsuccessful   Three of the 4 lots of *A aegypti* tested may be dismissed from consideration, as they evidently got little or no virus with their infective feeds   Five of the 8 mosquitoes tested in the remaining lot (Lot 5) contained virus, but normal mice bitten by insects of this lot on the 5th, 7th and 12th days after their infective feeds, and a normal lamb bitten on the 10th, 14th, 18th, 21st and 24th days (Table I), did not become infected   The mosquitoes usually fed avidly on either the mouse or the lamb offered   Their failure to transmit to mice could have been due to the fact that insufficient time had elapsed between the infecting feed and the latest attempt to transmit   This was probably not the case with the lamb, however, as this animal was bitten as late as the 24th day after the mosquitoes fed, and the tests for virus in the insects of this lot showed that the lamb was bitten on every occasion by 1 or more mosquitoes which were proved to contain virus   From this evidence we conclude that *Aëdes aegypti*, if it can transmit Rift Valley fever at all, is a very poor vector

Only 2 of 12 lots of *T fuscopennatus* which fed on infected mice could be regarded as suitable for transmission experiments   The other 10 lots either succumbed *en masse* before transmission could have been expected to occur, or

the tests by inoculation failed to show more than a very small percentage of the mosquitoes to have retained the virus   Even the remaining two lots could not be considered good, as only 16 of 71 tests for virus in Lot 15 and 10 of 98 in Lot 17 were positive   Although mosquitoes of these 2 lots bit normal mice on 6 occasions up to the 13th day after their infective feed, they failed to transmit the virus, possibly because of an inadequate period of incubation   The 6 bitings of a normal lamb by Lot 15 (Table I) were all by the same mosquito, which contained no virus when it was found dead on the 38th day   A test for virus in a mosquito of Lot 17 found dead on the 29th day was positive, so that there was at least one infected insect in the lot each time the mosquitoes bit the lamb up to and including the 27th day   Whether or not the lamb was ever bitten by a mosquito containing virus cannot be stated   The mosquitoes that bit the lamb on the 29th and 31st days gave negative results in tests for virus

No tests for the presence of virus were made in 2 of the 3 lots of *T uniformis* which had fed on infected mice, and no mosquitoes of these 2 lots bit normal animals during the maximum period of 12 days that any of them lived   None of the mosquitoes of the other lot were exposed to a lamb, and none could be induced to take blood again from a mouse   All were dead by the 14th day   Fifteen mosquitoes of this lot were tested individually for virus as they died, and it was found to be present in 6 of these (Table IV)   The longest period of demonstrated retention of the virus was 13 days

Susceptibility tests on animals used in unsuccessful transmission

Experiments —The lambs employed in unsuccessful attempts at transmission were tested for susceptibility at the end of the experiments, either by inoculation with fully virulent pantropic virus or by serum protection tests   None was immune   All the mice bitten by mosquitoes, other than the 2 previously mentioned as having been infected, were given intraperitoneal test inoculations of virus at the end of the experiment   All were shown to be fully susceptible   It is therefore known that the 4 transmissions which we have described were the only ones that occurred

Multiplication of virus in mosquitoes

No tests were done with the express purpose of determining whether Rift Valley fever virus multiplies in any of the species of mosquitoes included in our experiments   Nevertheless, the tabulation of the results of inoculation tests with mosquitoes of the most satisfactory lots of each species, by intervals between infective feed and test for virus, gave information on this point which may be significant (Table V)   It was seen that a high percentage of mosquitoes of the *E chrysogaster* group harboured virus throughout the experiments   This does not prove that the virus multiplies in the group , but if it does not multiply, it must be exceedingly well maintained   That it actually does multiply in these mosquitoes is suggested by their ability to transmit the virus, but only after an extrinsic incubation period of 19 or 20 days

Among the mosquitoes of the 2 species of *Taeniorhynchus*, on the other hand, the percentage containing virus was highest in the first 10 days after the infective feeds and then declined   This seems to indicate that, although a small proportion of *Taeniorhynchus* may maintain the virus for several weeks, in most mosquitoes of the species tested the virus slowly dies off in the insect without multiplying

TABLE V —*Summary of Results of Tests for Virus in Better Lots of Each Species of Mosquitoes by Intervals of* 10 *Days after Infective Feeds*

Interval between infective feed and test (days)	A aegypti, Lot 5		E chrysogaster group, Lots 1, 3, 7 and 11		T fuscopennatus, Lots 15 and 17		T uniformis, Lot 19	
	Tested	Positive	Tested.	Positive	Tested	Positive	Tested	Positive
1–10	2	1	22	16	117	22–1†	11	5
11–20	1	1	36	31	39	2	4	1
21–30	5	3	29	21–1*	11	1	0	
Over 30	0		1	1	3	0	0	

* 21 individual mosquitoes and 1 pool of 2 were positive, so that 22 or 23 contained virus
† 22 individual mosquitoes and 1 pool of 2 were positive so that 23 or 24 contained virus

The *Taeniorhynchus* mosquitoes used in these experiments took their initial (infective) feeds well   Only mosquitoes which showed no visible blood were exposed to infected animals   The immobilized mouse could then be left in the Barraud cage unattended, and the mosquitoes which had taken blood could be easily identified when inspected a few hours later   A high percentage of those exposed to infected animals obtained blood within a few hours   However, it was only with difficulty and the exercise of considerable patience that many of them could be induced to take blood again for a transmitting feed   Moreover, the mosquitoes of this genus did not thrive well in captivity (Table IV), and it was difficult to keep them alive long enough to carry out the necessary procedures   Whether these mosquitoes are short lived in nature, or are ill suited to the environment of the laboratory, is unknown, but it seems possible that their aversion to repeated blood meals is a characteristic of the genus

The *A   aegypti* tested were too few in number to permit conclusions with regard to multiplication of the virus in this species

Entomological note on the E   chrysogaster *group in the Kitinda area*

The *Eretmapodites chrysogaster* Graham group mosquitoes used in the transmission experiments were wild females caught in lake-side forest at Kitinda, about 2 miles west of Entebbe   The *E   chrysogaster* group as defined by Edwards (1941) contains 5 species , no wholly reliable characters are known by which the females of the group may be separated one from another   For the determination of the actual species used it was, therefore, essential to examine males derived from the same locality   Of 40 wild males collected there between December 11, 1947 and January 17, 1948, 37 were *E   chrysogaster* Graham and 3 were *E   intermedius* Edwards   It was considered possible, however, that wild females caught biting in the same locality might be of different species, so females were collected for egg-laying in order that males might be bred from them for examination   They were repeatedly offered blood meals in the laboratory   Those which fed were kept at about 24° C for 48 hours in Barraud cages covered with damp cloths, and were allowed access to 5 per cent glucose solution   They were then isolated individually in glass tubes (15 × 85 mm) lined with moist filter-paper and plugged with cotton   The tubes were stored at 30° C until eggs were laid or the females died   Batches of eggs laid by individual females were hatched separately , the larvae were mainly fed with appropriate instar larvae of *Aedes (S) aegypti* from a stock culture   *Eretmapodites* males hatching from these cultures were then examined to determine the identity of the original female which laid the eggs   In all, 608 females were collected between November

10, 1947 and January 19, 1948, and of these, 139 laid eggs However, a considerable proportion of these egg batches failed to hatch, and only 48 were successfully reared through to yield adult males for examination, i e, of the original 608 females, only 48, or 7 9 per cent, were identified by the male characters of their progeny However, all these 48 were found to be *E chrysogaster* Although the proportion definitely identified is small, the fact that all were *E chrysogaster*, taken together with the great preponderance of this species among the wild males, indicates that the *Eretmapodites chrysogaster* group population at Kitinda is composed almost entirely of *Eretmapodites chrysogaster* Graham itself, with only a small component of *Eretmapodites intermedius* Edwards

We are indebted to Mrs E C C van Someren for the determination of all but 18 of the terminalia examined, the remaining 18 have been identified by comparison with the material determined by her, and were all *E chrysogaster*

DISCUSSION

The circumstances of the outbreaks of Rift Valley fever affecting humans and domestic animals on farms in Kenya (Daubney and Hudson, 1931, 1933), and those of the outbreak in the Semliki Forest in Bwamba County, Uganda (Smithburn, Haddow and Gillett, 1948), were sufficiently different to indicate that the vector insects probably were not the same The country concerned in Kenya is open table land at an altitude of about 5000 feet, whereas the affected locality in Uganda is uninhabited virgin forest at an altitude of about 2500 feet Daubney and Hudson (1933) found that the protection of susceptible animals from mosquito bites during the hours of darkness alone sufficed for almost complete prevention of the infection, and this was strong evidence in favour of a night-biting vector On the other hand, all the mosquitoes from which virus was isolated in the Semliki Forest outbreak were taken on human baits during the hours of daylight Furthermore, the mosquitoes here incriminated as the probable vectors in that outbreak, the *Eretmapodites chrysogaster* group, are, in Uganda, day-biting insects which are not commonly taken in night catches Finally, it seems most improbable that this sylvan mosquito could propagate in the open country where the Kenya epidemics occurred

The aforementioned facts, plus the apparent demonstration of virus in wild-caught *Taeniorhynchus fuscopennatus* in Kenya (Daubney and Hudson, 1933), caused us to become interested in the genus *Taeniorhynchus*, most, if not all, of the members of which are night-biting mosquitoes In the original paper of Daubney and Hudson (1931) this mosquito was called *Taeniorhynchus brevipalpis*, while in a subsequent communication (1933) it was designated *Mansonia fuscopennata* Throughout the present paper we have used the terminology of Edwards (1941) The 2 species studied here, *T fuscopennatus* and *T uniformis*, have not yet been ruled out as possible vectors owing to the difficulty encountered in keeping them alive and in inducing them to take blood at any time after the first (infecting) feed Nevertheless, the tests for virus in mosquitoes of these 2 species showed a low percentage infected, and an apparent decline in the percentage containing virus with increasing passage of time after the infecting feed Neither *T fuscopennatus* nor *T uniformis* has, to our knowledge, ever been incriminated as the vector of any disease However, another member of

this genus, *Taeniorhynchus (Mansonioides) africanus* Theo , has been shown to be capable of transmitting yellow fever (Philip, 1930), and this or some other as yet untested species of the genus may be capable of transmitting Rift Valley fever

The occurrence of Rift Valley fever in the Semliki Forest, doubtless involving as host(s) some species of wild animal, the isolation of the causative virus from sylvan mosquitoes, and the subsequent experimental transmission of the disease by insects of one of the species from which the virus was isolated, not only places Rift Valley fever among the diseases which can be transmitted by insects, but associates it as well with the increasing group of diseases having cycles of infection which do not include man or domestic animals

While the experiments here reported do not incriminate a single species of mosquito as a vector of Rift Valley fever virus, they do show that at least one of the *E chrysogaster* group can transmit it   The mosquitoes of this group are so closely related and so alike in their habits that it seems possible that the whole group may possess vector potentialities

SUMMARY

Rift Valley fever virus was transmitted experimentally from mouse to lamb, from mouse to mouse, from lamb to lamb, and from lamb to mouse by the bites of mosquitoes of the *Eretmapodites chrysogaster* group   The success of these experiments indicates that these mosquitoes, which were included in the *Eretmapodites* spp from which the virus had previously been isolated, were probably responsible for the transmission of the virus in the Semliki Forest in 1944   The period of incubation in the *E chrysogaster* group was 19 or 20 days at 30° C

Attempts to transmit the virus with *Aedes aegypti* and with 2 species of the genus *Taeniorhynchus* were unsuccessful   It seems probable that the former cannot transmit this virus, but the experiments with *Taeniorhynchus* were inconclusive owing to technical difficulties   The virus may survive for a number of days in these mosquitoes, and it is possible that one or another species of this genus can serve as a vector

The results seem to place Rift Valley fever definitely in the group of insect-borne virus diseases

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FACTORS INVOLVED IN INFLUENZA HAEMAGGLUTINATION REACTIONS

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THE original object of our investigation was the study of any fluctuations of serum antibody level which might occur in individuals during inter-epidemic periods   The scale of the intended survey and the necessity for detecting relatively small antibody variations determined the choice of the haemagglutination test for our purpose   It was recognized that this test is complicated by variables pertaining to influenza viruses, chicken red blood cells and serum constituents other than specific antibodies, but the claims of previous workers and the precision of measurement attainable by means of a photo-electric densitometer encouraged the hope that variables might be sufficiently controllable to allow reasonably accurate antibody assays   The end in view has not yet been achieved, but unsuspected factors have been brought to light which may have an important bearing in work based upon the haemagglutination reaction

MATERIALS AND METHODS

Haemagglutination tests

The technique employed was essentially that of Hirst and Pickels (1942) in which the degree of fowl red cell agglutination occurring in test mixtures is measured by the amount of light transmitted through the mixtures from a light source of constant intensity on to a photo-electric cell   Instead of a micro-ammeter we used a sensitive mirror-galvanometer with scale readings from 0 to 100 and the light intensity was standardized at the beginning of each test so that 50 per cent of the test mixture concentration gave a galvanometer reading of exactly 50   Thus the reading 50 invariably corresponds to 50 per cent haemagglutination which has been adopted as the end-point in all titrations Provided that galvanometer readings above and below 50 are obtained determination of the virus or serum dilution corresponding to this end-point is a matter of simple calculation   Titres are expressed throughout as the reciprocals of such dilutions

For all serum titrations a standard dose of 4 agglutinating doses (4 AD) of virus was employed   To obtain this dose all virus preparations to be used in a test were titrated immediately prior to the test   Such preliminary titration is absolutely essential because of the variability of red blood cells of different fowls (Stuart-Harris 1943   Stuart-Harris and Miller, 1947) and the rapid decline of sensitivity of red blood cells during storage (Miller and Stanley, 1944) both of which phenomena we have amply confirmed   Moreover, the responses to heat treatment of different batches of a virus or even of the fluids from different eggs of the same batch may be far from uniform

Viruses

Influenza viruses A (PR8 strain), B (Lee strain) and Swine (Shope strain 15) were maintained by serial egg passage using the allantoic route of inoculation At the beginning of the investigation batches of each of the viruses were laid down in cold storage (— 20° C) to serve as reference standards , in each case pooled allantoic fluid virus was distributed in convenient quantities in sealed ampoules   These stored preparations are referred to as " stock " viruses ' Fresh " virus preparations were also used , they consisted of allantoic fluid harvested usually within 24 hours of the test, but sometimes a few days previously, and kept at approximately 4° C   Gross particles were removed from virus fluids by centrifugation

Sera

Immune horse sera to each of the viruses were distributed in sealed ampoules and kept at — 20° C as " stock ' sera   The A and B sera were reconstituted from freeze dried preparations kindly supplied by Dr C H Andrewes , the Shope serum was from a horse immunized in 1934 and stored since that time at approximately 4° C

Human sera were obtained from volunteers   If not required fresh they were inactivated in sealed ampoules at 56° C for 30 min and stored at — 20° C without addition of any preservative

Relevant details in respect of sera of other species are given in the text and protocols

Cholera enzyme

A potent enzyme preparation was obtained from agar cultures of *V cholerae* by the method of Burnet and Stone (1947), and was purified by adsorption on, and elution from, human red blood corpuscles   Crude filtrates were quite unsuitable for our purpose because of their high content of proteinase, which caused destruction of specific serum antibodies   The red blood cell eluate, however, showed no proteinase activity by the azocoll test (Oakley, Warrack and van Heyningen, 1946), although about two-thirds of the original receptor destroying enzyme (R D E) activity was retained   The eluate was distributed in ampoules in 0 5 ml quantities and freeze dried

EXPERIMENTAL

1   *Virus Degradation as a Factor in Serum Antibody Titration*

Instability of virus at — 20° C

Both the infectivity and the haemagglutinating activity of influenza viruses decline rapidly at room temperatures and more slowly at 4° C , but our reference stocks had been laid down on the assumption that virus kept in the frozen state at — 20° C would remain stable   Certain unexpected results obtained with the stock viruses led to a number of comparative titrations which are summarized in Table I   All tests at a stated period of storage were done in parallel with the same red cell suspension, so that titres in each vertical column of the table are comparable whereas differences along horizontal rows are of course without

any significance   It is evident that freezing and storage at − 20°C result in appreciable degradation of the PR8 virus, some of which occurs during the actual freezing process   Miller and Stanley (1944) found that virus remained stable for several months at 4° C , more stable in fact than when kept at − 70° C We have been unable to confirm this, for in our experience steady degradation has always occurred at 4° C , and some experiments recorded later provide evidence that the change is not only a question of reduction of haemagglutination activity but also of qualitative behaviour   It is probable that such discrepancies are due both to strain differences and to differences in physical states of the virus-containing fluids

TABLE I —*Instability of Influenza Virus PR8 at − 20° C*

Temperature ° C of		Haemagglutination titres after various periods of storage				
Freezing	Storage	30 min	15 days	32 days	32 days *	9 months
−76	−76	561	320			364
−76	−20			160	281	
−20	−20	444	160	134	101	269
	4	501	403	160	222	180
	R T	493				

* Glycerinated virus

Heat inactivation of virus

Francis (1947) showed that many sera contain a substance which inhibits haemagglutination by killed influenza B viruses but not when living virus is used   Thus, the apparent antibody titre of a serum may be much higher against killed virus than against living virus—an effect which we refer to as the Francis phenomenon or Francis effect   It is now generally accepted that the phenomenon is due to a heat-labile virus enzyme which destroys red cell receptors and which also rapidly destroys the Francis inhibitor   In early work with the PR8 influenza virus we encountered the same phenomenon   Whilst it is probable that the Francis inhibitor substance is effective against A viruses as well as against B viruses, there is, as yet, no proof of this and, as will be shown later, sera differ qualitatively in respect of their inhibitory properties, indicating the existence of several inhibitory substances of the Francis type   All the substances in sera effective against killed PR8 virus are therefore referred to as " alpha inhibitor," with full recognition of the likelihood that future work will lead to their separation and characterization

Because of the presence of such non-specific inhibitory substances in many sera, an analysis of the factors concerned in antibody titration demands the use of " inactivated ' viruses capable of revealing their presence   Inactivation has usually been by heating at 56° C for 30 min   but occasionally additional short treatment at 60° C has been used   Different virus strains have different degrees of susceptibility to heat (Salk, 1946), and the necessary destruction of virus enzyme may sometimes be achieved without any reduction of haemagglutinin activity (Briody, 1948)   In the present study, however, the viruses employed have invariably suffered a large reduction of haemagglutination titre on heating to 56° C for 30 min , indeed the Shope virus has always been completely inactivated by such treatment

PR8 virus has shown a striking variability in behaviour from one experiment to another, so that the degree of haemagglutinin reduction was never predictable , it appeared to depend not only upon the initial titre of the untreated virus, but also upon the age of the virus preparation since harvesting and possibly the sensitivity of the red blood cell suspension employed   Thus, stock virus usually suffered a 50 to 75 per cent reduction, whilst freshly harvested allantoic fluids often suffered less than 25 per cent   These points are illustrated in Table II

TABLE II —*The Effect of Heat Treatment, 56° C for 30 min , on Virus Haemagglutination Activity*

| Virus | Haem titres | | |
	Untreated	Heated	% Decrease
PR8	88	34	61
,,	131	38	71
,,	160	40	75
,,	400	148	63
,,	184	56	70
,,	160	80	50
,,	92	63	32
,,	90	81	10
,,	84	54	36
,,	435	218	50
,,	226	135	40
,,	123	55	55
Lee B	125	53	58
,,	188	124	34
,,	160	110	31
,,	180	106	41
,,	142	88	38
,,	80	70	12
Shope	114	0	100
,,	217	0	100
,,	213	0	100

More important than this variability of haemagglutinin reduction is the possibility that the heat treatment may occasionally fail to inactivate completely the virus enzyme which destroys alpha inhibitor, so that the presence of the inhibitor in a serum is masked   The temperature and time of inactivation employed, namely 56° C for 30 min , have been generally accepted as entirely sufficient for the purpose and Briody (1948) found that a still lower temperature sufficed, but doubt arose when a human serum showed no Francis effect on one day, but did so to a high degree when retested a few days later with older virus from an earlier egg passage   To settle the matter the serum was then titrated against the two virus fluids at the same time and with the same reagents   The previous results were confirmed, although the " fresh " virus was at the time of test already 4 days old   The experiments are summarized in Table III

The titration of the standard PR8 serum against living and heated PR8 virus consistently failed to show the Francis phenomenon   This was not sur-

TABLE III —*The Effect of Different Batches of Virus upon the Results of Serum Titrations*

Expt No	Date	Virus batch	Titres of virus		Titres of human serum " MS " against 4AD's of	
			Untreated	Heated	Untreated virus	Heated virus
1	18/vi/48	PR8/AL 44	726	415	65	69
2	21/vi/48	PR8/AL 42	190	98	60	435
3	22/vi 48	(PR8/AL 44	433	380	56	77
		(PR8/AL 42	186	127	70	>1280

prising, for some sera are devoid of the non-specific inhibitor substance   Repeated tests, however, with these stock reagents brought to light a phenomenon exactly the reverse of that of Francis, namely, that some sera exhibit a lower titre of haemagglutination inhibition against heated virus than against fresh living virus, especially if the latter is of high titre   With PR8 standard serum the differences were never large, but the consistency of the results of very many experiments indicated significance

A similar difference was obtained in each case with a number of immune ferret sera   With one of them (F 324, Table IV) a four-fold difference of antibody content was apparent according to whether untreated or heated virus was employed for the titration   On the other hand, a fresh normal ferret serum, normal rabbit sera, and all fresh human sera tested have given pronounced Francis effects

TABLE IV —*Effect of Virus Degradation upon Serum Antibody Titres*

Serum	Serum titres with 4 AD s of PR8 virus		Per cent decrease
	Untreated	Heat killed	
PR8 stock	370	253	32
,,	403	238	41
,,	372	236	36
,,	280	235	16
Immune Ferret 255	1920	1285	33
319	2358	1600	32
, 320	2751	1600	42
321	>3200	2436	>24
322	2540	1942	24
323	>3200	2580	>19
324	2540	580	77

This reduction of apparent antibody titre may be due partly to the increased bulk of virus protein contained in the 4 agglutinating doses of virus used, for the reduced haemagglutinin titre of heated virus necessitates the employment of larger quantities of virus fluid than in the case of untreated virus of high titre If virus bulk is indeed a factor of importance the proportions of living and dead virus in any preparation used for a serum antibody titration will affect the result, no matter how carefully the preliminary titration of the virus for the estimation

of 4 agglutinating doses has been carried out   This is supported by the results of serum titrations in parallel against mixtures of varying proportions of untreated and heated virus   As the proportion of heated virus is increased the serum titre steadily declines until not enough living virus is incorporated in the mixture to supply a critical dose of inhibitor-destroying enzyme   At this point sera containing alpha inhibitor show a sudden rise of titre, while those devoid of inhibitor show still further decline

The behaviour of the ferret sera, shown in Table IV, however, indicates that virus bulk cannot be the only factor concerned, because of two sera tested at the same time and with identical titres against living virus, one of them (F 324) shows over 75 per cent reduction, the other (F 322) less than 25 per cent   Thus the difficulty cannot be resolved by estimating serum antibodies in terms of the ratio of activity between the test serum and a standard serum   The ratios of the ferret sera in question would obviously depend upon whether untreated or heated virus were employed, and possibly, in the former case, upon the proportions of living and dead virus present   Which of the ratios would be more nearly indicative of the true *in vivo* virus neutralizing power it is impossible to say without further information from extensive correlation studies

2   *Haemagglutinin-inhibitory Substances of Normal Sera*

Species differences

The investigations reported above raised the suspicion that inhibitory substances of the Francis type in sera might affect results of antibody titrations even if fully active living virus were used , also that such substances might play some part in host resistance against natural infections with viruses of the influenza group   Investigations of " normal " human sera are complicated by the fact that, in addition to non-specific inhibitory substances, most of them contain specific virus-neutralizing antibodies which are believed to reflect previous influenza infections, either overt or sub-clinical   The sera of several other animal species were therefore examined   All were tested against both A and B influenza viruses, living and heat killed , in addition some were tested against swine influenza virus, but in this case only living virus could be used because of the complete destruction of its haemagglutinins by heating at 56° C   for 30 minutes (Table II)   Representative tests are recorded in Table V

TABLE V —*Inhibition of Virus Haemagglutination by Normal Sera of Various Species*

Serum	Serum titres with 4 AD's of viruses				
	PR8 living	PR8 killed	Lee B living	Lee B killed	Swine living
Guinea-pig	147	267	241	926	<40
Rabbit	32	236	61	370	<20
Mouse	76	46	83	83	<20
Rat	28	40			
Ferret	50	92	30	1085	
Horse	0	380	0	320	
Sheep	64	287	72	1024	>32
Chicken	60	202	20	587	21
Plaice	20	640	25	>1280	<20

Only single samples of sheep, chicken and plaice sera were available, but with the other species listed several individual sera were tested, all of which behaved like the representative recorded in the table It is clear that most species possess inhibitory serum constituents of the Francis type, effective against both A and B influenza viruses Differences in the magnitudes of the Francis effects obtained in different experiments are entirely without significance, for this largely depends upon the particular batch of virus used , indeed, with freshly harvested virus of high titre, the effect may be abolished altogether (cf Table III) The absence of the phenomenon in the cases of mouse and rat, however, is almost certainly due to a true lack of this particular type of inhibitory substance , sera of these species never show the effect, even when tested against virus batches particularly suitable for the purpose The obvious inference is that other inhibitory substances of different type exist This is also indicated by the fact that the sera of most species possess appreciable inhibitory power against living viruses of both types A and B , in curious contrast is the lack of similar activity against Swine influenza virus

Amongst the species so far investigated the guinea-pig appears to occupy a unique position Every guinea-pig serum tested has given remarkably high titres against living viruses, titres indeed of the same order as those given by adult human sera which contain specific antibodies This led us to test three guinea-pig sera for virus neutralizing antibodies in mouse neutralization tests in parallel with sera of other species The usual method of titrating serial dilutions of serum against a constant amount of virus containing many lethal doses cannot be expected to reveal small amounts of antibody , we therefore tested various dilutions of virus filtrate mixed with equal quantities of serum The experiments are summarized in Table VI All three guinea-pig sera showed evidence of considerable virus neutralizing power , one of them neutralized over a thousand minimal infective doses of virus In Experiment 3 a sample of the guinea-pig serum inactivated by heating at 56° C for 30 min was also tested The heat treatment caused almost complete abolition of neutralizing power, a result directly contrary to what was expected in view of the effect of inactivation upon haemagglutination inhibition described below One obvious possibility is that complement may be required for the *in vivo* neutralization, although, so far as is known, complement does not participate in the neutralization of viruses by their specific antibodies This aspect of the work is under further investigation, and will be more fully reported in a subsequent paper

Effect of heat inactivation of sera

It is customary to inactivate by heat treatment all sera intended for antibody titration The original purpose of this was, of course, destruction of complement which would obscure results of complement-fixation tests , in addition it provides some safeguard against bacterial contamination The possible effects of such heat treatment upon the anti-haemagglutination titres of immune sera appear to have been overlooked whilst in the case of normal sera published reports are directly contradictory (McCrea, 1946 , Hirst, 1948) This is not surprising in view of the complexity of the factors involved The experiments summarized in Table VII confirm the existence of quantitative differences in respect of alpha inhibitor between various animal species, but they also reveal qualitative

TABLE VI.—*Mouse Neutralization Test—Neutralization of Influenza Virus by Guinea-pig Sera*

Expt No.	Serum 1/2 dil	Dilutions of PR8 virus filtrate					
		1/20	1/200	1/2 × 10³	1/2 × 10⁴	1/2 × 10⁵	1/2 × 10⁶
1	Guinea pig A	0 D	0 0 D	0 0 +	0 0	0 + +	0 0 0
	Horse	+ D	+++ +++	++ + +	0 ± +	0 + +	0 0 0
2	Guinea pig B	+	+	0 0 0	0 0 0	++ 0 0	0 0 0
	Horse	++ +	+ D	D ++ +	+++ +++ +++	+++ ++ ++ 0	0 0 0
	Rat			++ ++ ++	+++ +++ +	++ ++ 0	0 0 0
	Rabbit			++ ++ ++	+++ +++		
3	Guinea pig C	+++ +++ +++	+ ± 0	± 0		+ +	
	Guinea pig C Inactivated D	D	D D D	+++ +++ ++ D	+++ +++ ++	+++ ++ ++	+ +
	Horse			+++ +++ +	+++ ++ ++	+	

D = Mouse died with almost complete lung consolidation
+++, ++, +, ± indicate diminishing degrees of lung involvement

differences   In tests against living virus the effect of heat inactivation of the sera of all species is to reduce their inhibitory power   Tests against killed virus, however, separate the species into distinct categories   Some, like mouse, yield sera which always suffer reduction of titre on heat inactivation   others, like rabbit, have sera which may show either reduction or slight increase of doubtful significance, whilst guinea-pig sera invariably show a large increase which may amount to as much as a tenfold rise   This peculiar property of guinea-pig sera cannot be due merely to an exceptionally high content of alpha inhibitor because rabbit sera containing just as much may show the contrary effect

TABLE VII —*Heat Inactivation, 56° C for 30 min   of Normal Sera of Different Species*

Serum	4 AD s living PR8 virus with—		4 AD's killed PR8 virus with—	
	Fresh serum	Heated serum	Fresh serum	Heated serum
Guinea-pig 1	178	213	470	5120
„ 2	172	121	1280	3482
„ 3	286	181	806	5120
Mouse 1	59	0	42	0
, 2	76	0	46	0
, 3	>128	41	111	18
Rabbit 1	32	20	236	177
„ 2	67	32	415	237
, 3	54	34	1350	1670
4	64	45	1561	1881
Ferret	50	48	92	119

The effect of heat inactivation upon adult human sera is quite unpredictable As with other species it always results in reduction of titre against living virus, but against killed virus there may be reduction, increase or no significant change Samples obtained from an individual donor at different times usually all show the same type of effect, but this is not invariably the case   There is also some evidence that the demonstration of increased activity on heat treatment is partly dependent upon the degree of susceptibility to virus agglutination of the chick cells employed in the test   A full explanation of these peculiarities of human sera has not been found but it seems probable that they depend upon the relative concentrations of specific antibodies and non-specific inhibitor substances Whilst in the absence of further antigenic stimuli the concentration of specific influenza antibodies in a person's serum remains fairly constant over a long period the concentration of other haemagglutination inhibitor substances may undergo considerable fluctuations

Fluctuations of serum inhibitor substances in human volunteers

During the period October 1947, to May, 1948, 21 volunteers, chiefly medical students, were bled at intervals of one to two months   From each sample the serum was separated inactivated by heating at 56° C for 30 min in a sealed ampoule and stored at − 20° C until required for test   All the samples from each individual were tested in parallel at one and the same time against both

living and heat-killed PR8 virus Most of the volunteers gave four samples, but some gave only two or three The results of all the tests are tabulated in Table VIII A surprising degree of fluctuation is shown by most of the sets of sera As was to be expected, the serum changes are best revealed by tests against killed virus, for they probably consist chiefly of fluctuations in concentration of alpha inhibitor Titre differences up to 30 per cent may be due to the experimental error of the test and are without any significance, but no less than 16 of the 21 volunteers yielded sets of sera giving over 50 per cent differences, and 11 sets show over 100 per cent differences Still more surprising is the fact that 9 sets of sera show over 50 per cent differences in the tests against living virus None of the volunteers suffered any illness resembling influenza during the sampling period, so that there appears to be no escape from the conclusion either that alpha inhibitor may affect the results of serum antibody titrations even when living virus is used, or that other serum constituents, more akin to the specific antibodies and not destroyed by the virus enzyme, may increase and decrease from time to time quite independently of any specific antigenic stimulation Possibly both explanations are true, but the hypothesis that serum titres in the standard type of test with living virus may be partly

TABLE VIII —*Serum Titre Fluctuations in Human Volunteers*

Serum donor	Living PR8 virus Serum samples						Killed PR8 virus Serum samples					
	1	2	3	4	H/L	% Inc	1	2	3	4	H/I	% Inc
S A—	139	138	144	133	1 1	8	181	187	170	148	1 3	26
E H—	213	181	177	171	1 2	24	192	177	235	168	1 4	39
C E—	>640	448	380	497	1 7	68	1228	392	240	413	5 1	411
D M—	93	52	61	55	1 8	78	320	95	127	77	4 2	315
M W—	40	<40	<40		1 0		452	190	242		2 4	137
J M—	110	82	127	85	1 5	54	359	307	250	247	1 4	40
E L—	320	121	242	132	2 6	164	2560	508	1280	761	5 0	403
S B—	94	106	68	94	1 6	55	1689	1689	537	1280	3 1	214
M P—	128	95	45	75	2 8	184	4304	4362	830	1280	5 3	425
L G—	50	52	51		1 0	4	1016	2152	735		2 9	192
P T—	87	62			1 4	40	2228	1076			1 1	107
W C—	58	58			1 0		254	137			1 9	85
D F—	196	174	168		1 2	16	351	320	233		1 5	50
V W—	72	85	101		1 4	40	195	320	452		2 3	131
F K—	118	112	132		1 2	17	320	261	292		1 2	22
E M—	160	101	88	89	1 8	81	640	735	462	538	1 6	59
E B—	70	52	54		1 3	34	282	274	288		1 -	5
C M—	103	82	62	109	1 8	75	970	508	493	707	2 0	98
J H—	114	130	126	116	1 1	14	1280	951	844	485	2 6	163
M M—	262	265	234		1 1	12	1902	1522	761		2 5	149
J D—	74	103	116	116	1 6	56	345	640	545	640	1 9	85

H/L = Ratio of highest and lowest titres
% Inc = Percentage increase of highest over lowest titre

determined by alpha inhibitor is strongly supported by the fact that, on the whole the significant fluctuations shown in the tests with killed virus are reflected by corresponding smaller fluctuations against living virus

The use of cholera enzyme in antibody titration

Because of the instability of living influenza virus and the unpredictable behaviour of freshly harvested batches the use of killed virus for antibody assay

would be preferable provided that all non-specific inhibitor substances could be removed from the sera to be tested   The possibility of achieving this by means of cholera enzyme has been explored   Since the completion of the work a recent publication by Mulder and van der Veen (1948) became available which reports the use of cholera enzyme for the same purpose but without giving details of the results of the treatment

A purified enzyme preparation was used which showed no proteinase activity by the azocoll test   Equal quantities of the enzyme in calcium acetate buffer and serum were mixed, incubated at 37° C for an hour and then heated at 56° C for 30 min to inactivate the enzyme   A control mixture of the serum and buffer solution was subjected to the same treatment in parallel   Preliminary tests showed that the buffer solution itself had no deleterious action on the inhibitory power of serum

The results of experiments on three human and three normal guinea-pig sera are shown in Table IX   They indicate that the method may prove satis-

TABLE IX —*Treatment of Sera with Cholera Enzyme*

Serum	Control or enzyme treated	Serum titres with 4 AD's PRS virus	
		Living	Heat killed
Human W S	C	42	109
	E	<32	<32
„ M W	C	53	456
	E	40	44
, Mar	C	64	254
	E	47	38
Normal guinea-pig 1	C	640	9122
	E	293	3880
, „ 2	C	160	3225
	E	48	806
„ „ 3	C	44	1762
	E	<40	320

C = Control sample   E = Enzyme treated sample

factory provided that the potency of the enzyme used is adequate for removal of all alpha inhibitor contained in the serum   With the three human sera this was the case for after the enzyme treatment titres against living and killed virus were not significantly different   These titres, however were consistently lower than the tires of the corresponding control samples against living virus   The differences may possibly have been due to the concomitant destruction of some of the true specific antibodies by residual proteinase in the enzyme preparation which was not detected by the azocoll test   It is however, much more probable that such reductions of titre represent the part played by alpha inhibitor in tests against living virus   The experiments with guinea-pig sera strongly support this view   Obviously the enzyme used was not potent enough to destroy all alpha inhibitor present in the guinea-pig sera, but in each case the effect of the enzyme treatment was to reduce titres against both killed and living virus by approximately the same proportionate amount

DISCUSSION

The importance of virus haemagglutination reactions is now well established for by their agency considerable light has been shed upon some of the fundamental problems of infection Their use, however, for the detection and measurement of serum antibodies is open to question For such purposes the desirability of using standardized and stable virus is obvious, but it is doubtful whether this is possible with living virus preparations, for these have been shown to vary from time to time and from batch to batch, not only as regards their haemagglutination titres, but also qualitatively in a manner which affects the inhibitory potency of sera The factor chiefly responsible for such instability may be the unpredictable and ever-changing proportions of living and dead virus In any case heat-killed virus is much more likely to be both stable and standardizable, but it can be employed for antibody assay only in the complete absence of non-specific inhibitory substances of the Francis type The possibility of removing such inhibitory substances from sera by treatment with cholera enzyme has been demonstrated by the few preliminary experiments reported, but before the method can be adopted rigorous proof is required that the treatment is without any deleterious effect upon specific antibodies, and that the titres of enzyme-treated sera obtained by haemagglutination show a close and constant correlation with titres obtained by mouse neutralization tests

The alpha inhibitor and probably other serum constituents of like nature participate in influenza haemagglutination reactions even when living virus is used, and under some circumstances they may apparently affect the results of antibody titrations This might be of little importance if all human sera contained approximately the same amount of non-specific inhibitors, or even if the serum of each individual remained constant Such, however, is not the case , in this respect animal species vary, different members of each species vary, and the serum of an individual may fluctuate from time to time The majority of the volunteers examined showed considerable fluctuations over a short period of a few months, in spite of the facts that the group was fairly uniform as regards age, social status and mode of life and that none suffered illness throughout the period of experiment It is true that in this small series the maximum effect upon the serum inhibition titres of any individual was only a 2 8-fold increase when living virus was employed for the titrations, but it is probable that much greater serum changes may occur from time to time as a result of non-specific stimuli of various kinds Indeed we have already obtained some evidence that febrile disturbances quite unconnected with virus infections may affect profoundly the concentration of alpha inhibitor in the patient's serum Thus the usual interpretation of titre differences between acute and convalescent samples of serum, in both diagnostic and epidemiological work, is open to question

Apart from their participation in haemagglutination reactions serum inhibitors of the Francis type may be of considerable importance It is difficult to believe that serum constituents capable of blocking the virus receptors concerned in the initial stages of cell invasion are without any influence upon the susceptibility of the host to infection In many virus diseases including influenza, the behaviour of individuals and the epidemiological patterns of outbreaks are not always explicable on the basis of levels and distribution of specific antibodies in the population at risk Non-specific factors which are completely

overshadowed in experimental laboratory work may well play a part in natural infections and may also be concerned in the determination of species susceptibility and resistance   It is in this connection that the *in vivo* neutralization of virus by normal guinea-pig sera may have special significance, though it is, as yet, quite uncertain whether their neutralizing power is connected with their exceptionally high concentration of alpha inhibitor   The two effects of heat inactivation, namely increase of haemagglutination inhibition titre against killed virus and loss of virus neutralization activity in mouse tests, suggest that serum constituents of different nature must be responsible for the two types of activity   Burnet and McCrea (1946) report similar high haemagglutination inhibition titres of normal ferret sera coupled with a virus neutralization activity which was peculiarly strain specific   In their case, however, the inhibitory substance was found by salt precipitation methods to be a part of the gamma globulin fraction of the serum proteins, and they assumed that the same component was responsible for the different types of activity   Obviously further investigations of the sera of different species by all available methods are indicated

SUMMARY

Influenza viruses suffer degradation by freezing and storage even at very low temperatures   Qualitative differences in different batches of virus may affect results of serum titrations

Immune sera devoid of non-specific inhibitor substances of the Francis type (alpha inhibitor) give lower inhibition titres against heat-killed influenza A virus than against living virus   It is suggested that the bulk of virus protein contained in the standard haemagglutinin dose of virus employed may be partly responsible for the phenomenon

The sera of all animal species tested were found to contain non-specific inhibitory substances   In addition to quantitative differences, species differ in respect of the qualitative behaviour of their serum inhibitors   Guinea-pig sera appear to be especially rich in such substances

Of three normal guinea-pig sera tested in mouse neutralization tests all showed virus neutralizing potency

Considerable fluctuations of the concentration of non-specific serum inhibitory substances occur from time to time in normal healthy human beings   Such changes may affect the results of influenza antibody titrations by the haemagglutination reaction even if living virus is employed

The removal of alpha inhibitor from sera by means of cholera enzyme may facilitate the accurate assay of influenza antibodies in human sera

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THE EFFECT OF HEAT UPON DIPHTHERIA TOXOID-ANTITOXIN FLOCCULES

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IT was shown by Glenny and Pope (1927) that the antigenic efficiency of toxoid-antitoxin floccules, as measured by the Immunity Index method, was improved by heating for 1 hour at 80° C Glenny and Waddington (1928) confirmed this observation, and showed that the filtrate from a heated batch produced immunity when injected into guinea-pigs Ramon, Legroux and Schoen (1931) suggested decomposition of the floccules on heating, with destruction of antitoxin, and solution of the more heat-stable toxoid Watson, Taggart and Shaw (1941) found that the solubility of floccules and the antigenic activity of filtrates increased with increasing pH level at which the heating was performed up to approximately pH 9 0, above which the activity decreased They tested the filtrates for toxoid content by means of the flocculation test using an enzyme-purified antitoxin , they were unable to obtain flocculation against other sera

In the experiments now to be described, the floccules under investigation were suspended in buffer solutions , between pH 6 2 and 7 5 sodium phosphate buffers (Sorensen) were used, and above pH 7 5, sodium borate-boric acid buffers (Palitzsh) The range below pH 6 2 was not used, because early experiments showed that no appreciable quantity of toxoid was freed on heating under these conditions Filtrates from heated samples were tested for free toxoid content by the flocculation test, at least two types of test being made on each sample, as described later This was done because the nature of the filtrates suggested that unreliable results might be obtained if any undestroyed antitoxin were present In agreement with the results of Watson, Taggart and Shaw, no flocculation as a rule could be obtained against unconcentrated sera, though in a few cases flocculation occurred at such a point that it was obviously indicating an incorrect value (e g more toxoid present than was originally contained in

the floccules), it was always possible, however, to obtain a result by blending the filtrate with a suitable volume of a toxin of known strength, and testing against an unconcentrated serum   Further tests were made against refined antitoxin, both by direct titration and by testing blends   It was sometimes found that the results obtained by different methods were discrepant, or that more than one zone of flocculation existed   When this occurred, a different antitoxin was used in further tests in order to determine which zone represented the true neutral toxoid-antitoxin reaction , in general the method of the blend test was the most satisfactory one

It is generally assumed that toxoid-antitoxin floccules consist of pure toxoid and antitoxin, but the abnormal flocculation reactions of these filtrates, either in the form of impossible values or more than one zone, suggests one of two possibilities   The first of these is that the floccules actually contain specific components other than toxoid and antitoxin, and the second that the presence of some undestroyed combined antitoxin causes flocculation in multiple proportions under certain conditions   The latter explanation gains some support in subsequent findings   While the testing of filtrates from most batches of heated floccules presented some difficulty, particularly those heated at pH 8 5 and 9 0, it was especially evident in filtrates from floccules prepared from refined antitoxin, which is more heat-stable than unconcentrated antitoxin   With one exception the floccules used in the experiments to be described were standardized in volume so as to contain approximately 120 Lf per ml of toxoid   The toxoid and antitoxin after flocculation was allowed to stand for 4 weeks, after which the floccules were washed several times with saline and suspended in buffer solutions

The effect of heating at different temperatures floccules prepared from the same materials in different proportions

Three batches of floccules were prepared, using one toxoid and one unconcentrated antitoxin   The first batch (1984) contained approximately 25 per cent less antitoxin than the Lf mixture, the second batch (1985) contained toxoid and antitoxin in equivalent proportions, while the third (1986) contained antitoxin in excess to about 25 per cent   Heating was carried out on 100 ml quantities of suspension of these floccules at 70° C , 75° C and 80° C for 1 hour at pH levels ranging from pH 6 17 to pH 9 0   Table I shows the titres of toxoid in Lf doses per ml found in the filtrates after heating, and it will be seen that in general the higher the temperature the more toxoid was freed, though there are indications that destruction may occur at 80° C above pH 8 5   The results confirm those of Watson, Taggart and Shaw in so far as the amount of toxoid present increased with increasing pH level, but in contrast to their findings more toxoid was recoverable under the same conditions from floccules prepared using excess toxoid than from floccules containing an excess of antitoxin   In order further to test this point, five batches of floccules were prepared using another toxoid flocculated with different amounts of a refined antitoxin   These batches contained 0 66, 1 0, 1 5, 2 0 and 2 3 units of antitoxin per Lf dose of toxoid   Portions of the floccules were suspended in buffer solutions and heated for 1 hour at 75° C , the results are shown in Table II   The essential findings in this experiment are the same as those shown in Table I, for the amount of

TABLE I —*Showing the Amounts of Toxoid Detectable in Filtrates from 3 Batches of Toxoid-Antitoxin Floccules after Heating for 1 hour at Different pH Levels*

Batch of floccules	1984			1985			1986		
Units of antitoxin per Lf toxoid	0 75			1 0			1 25		
					Temperature				
	70°	75°	80°	70°	75°	80°	70°	75°	80°
pH				Toxoid content of filtrates (Lf per ml)					
6 17	0	3	6	0	0	3	0	0	2
6 55	1	3	8	0	2	6	1	1	2
7 05	3	11	26	1	10	19	1	3	10
7 65	5	15	29	1	14	28	3	8	10
8 00	13	22	30	3	29	30	3	10	18
8 20	28	43	59	4	40	63	4	22	39
8 50	37	53	ca 50	29	ca 60	—	12	40	44
9 00	60	60	—	44	—	42	27	42	20

TABLE II —*Showing the Amounts of Toxoid Detectable in Filtrates from 5 Batches of Toxoid-Antitoxin Floccules after Heating for 1 hour at 75° C at Different pH Levels*

Batch of floccules	2110	2123	2124	2125	2126
Units of antitoxin per Lf toxoid	0 66	1 0	1 5	2 0	2 5
pH	Toxoid content of filtrate (Lf per ml)				
6 55	5	5	1	1	0
7 05	10	8	3	3	1
7 65	19	10	5	3	1
8 0	35	18	5	3	1
8 2	44	16	10	3	1
8 5	50	14	12	6	1
9 0	50	—	—	6	2

toxoid passing into solution at a given pH level decreases as the antitoxic content of the original floccules increased   This is in direct contrast to the results of Watson, Taggart and Shaw, who found that recovery of toxoid was greatest from floccules in which the antitoxin was considerably in excess

In all experiments, especially at 80° C , many of the suspensions of floccules underwent coagulation , those suspended in buffers at pH 8 2 or above frequently went into solution after heating for 10 or 15 minutes

A comparison of the antigenic efficiency of suspensions of heated floccules containing different amounts of toxoid in solution

Tests for antigenic efficiency were made by means of two equal doses of material injected subcutaneously into groups of 12 or more guinea-pigs at an interval of 28 days   The animals were bled 10 days after the second injection,

and the individual bleedings tested for antitoxic content by the guinea-pig intracutaneous method   Tests were made at approximately 2-fold differences, the lowest value tested being 0 001 unit   In all tables showing antitoxic response of guinea-pigs it has been necessary to record the median value rather than the geometric mean, because in some groups a proportion of the animals failed to produce any detectable antitoxin   The extent of the scatter of the antitoxic values is of importance in giving some indication of the group response to injection, but the figures obtained in these experiments are too extensive for present-day publication, and have therefore been summarized in the form of median values for each group   With increase in dosage or improvement in antigen scatter is reduced, though the relation between dose and response is not nearly so close for toxoid-antitoxin floccules as for other types of antigen such as toxoid or alum-precipitated toxoid

Table III gives a comparison of the antigenic efficiency of a number of suspensions produced by heating 100 ml quantities of a batch of floccules for

TABLE III —*Showing the Immunity Response of Guinea-pigs to 2 Injections each 0 1 ml of Toxoid-Antitoxin Floccules Heated at Different pH Levels*

Sample	pH	Free toxoid (Lf per ml)	Median antitoxic value (units per ml)
1	6 17	0	0 706
2	7 0	10	0 708
3	8 0	29	2 33
4	8 2	40	3 55
5	8 5	60	1 09
6	unheated control		0 0235

1 hour at 75° C at different pH levels   The floccules used had been prepared by mixing equivalent proportions of toxoid and unconcentrated antitoxin, and the supernatant liquids from the heated samples contained toxoid in solution in amounts ranging from 0 to 60 Lf doses per ml

All heated samples were considerably better antigens than the unheated floccules, including that heated at pH 6 17, in the filtrate of which no detectable toxoid was present   The antitoxic response was greatest in guinea-pigs injected with the sample containing 40 Lf per ml of free toxoid, and a titre of 29 Lf gave a better result than one of 60 Lf   It should be pointed out however that many tests using different dosage would be necessary to determine the optimum balance between free toxoid and undissolved floccules   In this experiment no guinea-pig produced less than 0 1 unit of antitoxin except those injected with the unheated control , only 3 out of 14 in this group produced more than 0 1 unit and 3 failed to reach 0 001 unit   The use of a lower dose might have suggested that suspensions containing less free toxoid were the better antigens, because in general low doses of precipitated antigens are more effective than low doses of soluble toxoids   In unpublished experiments done at these laboratories it has often been found that a reduction of the dose to one-tenth or one-hundredth of that prescribed under the Therapeutic Substances Act for the testing of toxin-antitoxin floccules (T A F) produces but little difference in the antigenic effect

A further comparison was made using a different batch of floccules in which refined antitoxin was used , the amount of antitoxin was 80 per cent of that required for neutrality The samples of floccules were heated for 1 hour at 70° C , and the free toxoid titres of the resulting suspensions ranged from 0 to 44 Lf per ml In the testing of antigenic efficiency the same method was used, but doses of 0 1, 0 01 and 0 001 ml were injected for each sample The results are given in Table IV, and show, as in Table III, that all the heated preparations

TABLE IV —*Showing the Immunity Response of Guinea-pigs to 2 Injections of Toxoid-Antitoxin Floccules Heated at Different pH Levels*

Sample	pH	Free toxoid (Lf per ml)	Median antitoxic value (units/ml) of animals injected with—		
			0 001 ml	0 01 ml	0 1 ml
1	6 55	0	0 050	0 141	0 251
2	7 65	2	0 0141	0 174	0 505
3	8 2	12	0 123	0 317	0 133
4	8 5	27	0 500	0 573	0 833
5	9 0	44	0 234	0 537	1 59
6	unheated control		—	0 0020	0 0913

were better antigens than the unheated floccules In general the antigenic efficiency increased with the amount of free toxoid in solution, but the samples heated at pH 8 5 and 9 0, with 27 and 44 Lf per ml respectively, appeared equally efficient The fact that the latter appeared slightly better than the former when a dose of 0 1 was used, but worse when 0 01 was used, if not due to animal variation, may be the result of the relative proportions of dissolved and undissolved material as suggested earlier, or to the physical condition of the remaining floccules favouring greater antigenic efficiency after heating at pH 8 5 than pH 9 0

Examination of the results with regard to median value and degree of scatter shows that the values obtained using Samples 1, 2 and 3 are sufficiently comparable to be collected together into a group B for each level of dosage , Samples 4 and 5 form another group, C, and the unheated control alone a third group, A The distribution of values arranged in this manner is presented in Fig 1, which gives the percentage of the total number of guinea-pigs exceeding certain values (log scale)

A comparison of the curves shows that all become steeper, owing to reduction in scatter, with increasing dosage As the dose is increased, the difference in antigenic efficiency of the samples also becomes far less apparent The most interesting facts, however, arise from a comparison of the actual antitoxic values reached by the majority of guinea-pigs in each group These can be briefly summarized as follows —

Group A3 (0 1 ml), 15 guinea-pigs , 13 3 per cent failed to produce antitoxin, but of the remainder, 73 per cent produced 0 04, 0 1 or 0 2 unit

Group B3, 39 guinea-pigs, no failures , 79 per cent produced 0 1, 0 2, or 0 5 unit

Group C3, 25 guinea-pigs, no failures , 78 per cent produced 0 5, 1, or 2 units

There is but little scatter in groups B and C at this dosage, and the range of values within which the majority fall is somewhat higher for C than B The
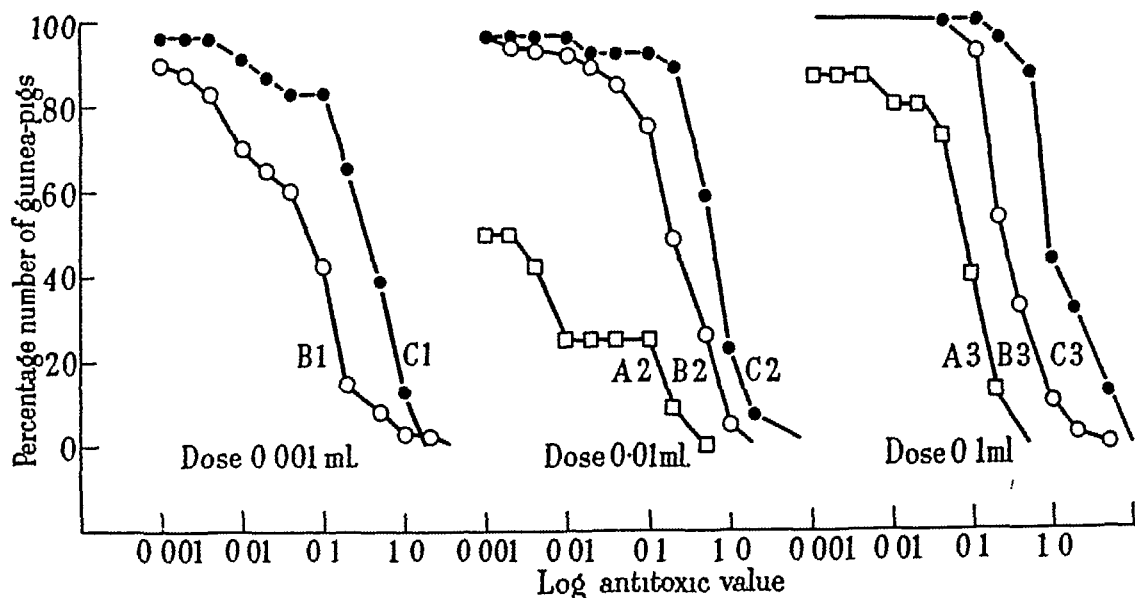
Fig 1 —Showing the percentage number of guinea pigs exceeding certain antitoxic values after two injections of unheated toxin antitoxin floccules (Curves A), heated toxin-antitoxin floccules Samples 1 to 3 in Table IV (Curves B), and Samples 4 and 5 (Curves C)

chief difference between A and B lies in the failure of 13 per cent of group A animals to respond , the majority of the responders produced values comparable with the majority of group B animals

Decreasing the dose to 0 01 ml , the difference between A and the other groups is more apparent   50 per cent of the 12 guinea-pigs (A2) failed to respond, and 33 per cent of the remainder had values of 0 1, 0 2 unit   Group B2, 65 guinea-pigs, 3 1 per cent failures, 71 per cent of the responders produced 0 1, 0 2, 0 5 unit   Group C2, 28 guinea-pigs, 3 6 per cent failures, 82 per cent of responders produced 0 2, 0 5, 1 unit   At this level of dosage, therefore, A is inferior to B and C for two reasons , there is a much larger percentage of non-responders, and a much smaller group with values equal to the majority of responders in B and C

Using the lowest dose (0 001 ml) the control sample A was not tested   In Group B1 10 3 per cent of the 39 guinea-pigs failed to respond, and 59 per cent of the remainder had values of 0 04, 0 1, 0 2 units, while in Group C1, 4 4 per cent of 23 guinea-pigs failed, and 69 per cent of the responders produced 0 1, 0 2, 0 5 units

These results show that the majority of those guinea-pigs which showed an antitoxic response produced values which fall within a small range, and this range scarcely altered even when the dose injected was increased or decreased 10 to 100-fold

In an attempt to determine the cause of the improvement in antigenic efficiency of heated samples whose filtrates contain no detectable toxoid, one such batch was centrifuged, the supernatant liquid removed, the floccules well washed several times with saline and resuspended to original volume   This suspension and the unwashed heated suspension were injected into guinea-pigs When two doses each of 0 1 ml were injected, there was no difference in the response of the two groups of animals, but using a dose of 0 01 ml the unwashed

suspension appeared to be slightly better , both preparations were superior to the parent unheated floccules   A single injection of 5 0 ml of the supernatant liquid produced low-grade immunity in another group of guinea-pigs, 8 out of 13 animals producing from 0 001 to 0 2 unit of antitoxin in 6 weeks, and the remaining 5 failing to respond   It appears probable that the increased antigenic efficiency of preparations containing no detectable toxoid is due to some physical change in the floccules during heating, as well as to the action of traces of toxoid undetectable by blend flocculation test, or of soluble toxoid-antitoxin   Support is lent to this idea by the results of the following experiment

The effect on antigenic efficiency of addition of heated dissolved toxoid-antitoxin floccules to unheated floccules

As has been stated previously, batches of floccules heated at 75° C or 80° C at pH 8 5 or 9 0 usually pass completely into solution   It appeared possible that the properties of heated floccule suspension might be reproduced by the addition of heated dissolved floccules to an unheated suspension   A batch of floccules was therefore heated at 75° C for 1 hour at pH 8 5, and the resulting solution found to contain 60 Lf doses of toxoid per ml   Portions of this solution were then added to measured volumes of a batch of unheated floccules in such quantities that the final concentration of toxoid in the mixtures was 5, 10, 20, 30 and 45 Lf per ml

All these mixtures therefore contained the same total nitrogen and the same amount of total antigen, though the proportions of floccules to dissolved floccules varied

The antigenic efficiency of the mixtures was tested by the usual method of two injections into guinea-pigs, and the results are given in Table V   These results should be compared with those in Table IV, because the batch of floccules

TABLE V —*Showing the Immunity Response of Guinea-pigs to 2 Injections of Toxoid-Antitoxin Floccules to which Dissolved Floccules had been Added*

Sample	Toxoid added (Lf per ml)	Median antitoxic value (units/ml) of animals injected with—	
		0 001 ml	0 01 ml
1	5	Under 0 001	0 0089
2	10	0 050	0 040
3	20	0 0036	0 034
4	30	0 016	0 063
5	45	0 080	0 040

used was the same for both experiments   The mixtures were all better antigens than the parent batch (unheated control, in Table IV), but none was nearly so efficient as any of the heated samples in Table IV , some failures to respond occurred in every group   This result strongly suggests that increased antigenic power due to heating is due not only to destruction of antitoxin with liberation of soluble toxoid, but also to some change in the remaining floccules, probably either in solubility in the tissues, stability or ease of dissociation

The effect of phenol in the heating of toxoid-antitoxin floccules

In a single experiment a batch of floccules prepared from refined antitoxin was divided into four portions treated as follows —

  (a) Suspended in buffer at pH 7 1, no phenol used
  (b) ,, ,, ,, pH 8 0, ,, ,, ,,
  (c) ,, ,, ,, pH 7 1, 0 5% phenol added
  (d) ,, ,, ,, pH 8 0, 0 5% ,, ,,

These suspensions were heated for 1 hour at 80° C and tested for antigenic efficiency   Portions of the filtrates were tested for toxoid content by the flocculation test, and it was found that within the limits of error of the test the same titre was obtained in the presence or absence of phenol for both pairs of suspensions   In the antigenic efficiency test, using 15 guinea-pigs for each sample, the presence of phenol during heating had no apparent effect at pH 7 1, but at pH 8 0 the sample containing phenol was definitely inferior to the control heated without phenol   It might well be expected that denaturation of toxoid would occur at pH 8 0 or higher in the presence of phenol, but further work is necessary before any final conclusion can be drawn

The effect of salt

It is well known that toxoid-antitoxin floccules show a certain degree of solubility in distilled water, but appear to be relatively insoluble in normal saline solution , they also go into solution at low pH levels (Glenny and Pope, 1927)   In order to facilitate recovery of toxoid by heat-treatment, reduction of the salt content of the suspending liquid should presumably be aimed at, in order to increase solubility, although at certain pH levels (above about pH 8 5) complete solution may readily occur on heating   If distilled-water suspensions of floccules are heated in glass containers, changes of pH are very liable to occur, and the conditions cannot therefore be controlled   Because of this, floccules used in the experiments described here have been suspended in buffer solutions free from sodium chloride   The pH level has thus been stabilized at the cost

TABLE VI —*Showing the Effect on Toxoid Liberation of the Addition of Sodium Chloride to Floccules Suspended in Buffer Solutions and Heated at 80° C*

pH	Toxoid content of filtrates (Lf/ml) recovered from floccules					
	A		B		C	
	With NaCl	No NaCl	With NaCl	No NaCl	With NaCl	No NaCl
6 55	10	18	6	6	2	3
7 05	22	40	10	8	2	4
7 65	31	50	14	15	3	5
8 0	30	52	16	31	4	6
8 2	53	110	26	55	7	9
8 5	30	120	21	50	8	16
9 0	—	ca 30	10	10	6	5
Units of antitoxin per Lf toxoid in floccules	0 66		1 0		1 5	

of the inclusion of certain electrolytes The effect of the addition of sodium chloride to the buffers has, however, been studied In these experiments portions of a batch of floccules were freed from salt by washing with distilled water, and suspended in buffer solutions , the companion sets were suspended in saline solution, which was drawn off and the floccules suspended in buffers containing 0 20 per cent sodium chloride Three batches of floccules were used, one, A, in which 0 66 equivalent of antitoxin was present, the second batch, B, one equivalent, and the third, C, 1 5 units of antitoxin per Lf dose of toxoid The results of heating these suspensions are shown in Table VI The presence of sodium chloride strongly inhibits the liberation of toxoid from the floccules, and in many instances the titre of toxoid found in the sodium chloride samples is of the order of half that in the corresponding controls

Total nitrogen estimations were done on these filtrates for the purpose of comparing the purity of the free toxoid in different series In every case the purest filtrates were obtained at pH 8 2, and the actual figures for purity of these samples in terms of Lf per mg nitrogen are as follows —

	Lf with sodium chloride	Lf without sodium chloride
Batch A	600	790
„ B	330	510
„ C	127	145

These figures show that the filtrates contain considerable amounts of soluble protein derived from the antitoxin constituent, because the purities of filtrates from Batch C are low and even those from Batch A are not especially high ; the more antitoxin was used in the preparation of the floccules, the less pure the resulting filtrates after heating, in terms of Lf doses per mg N This would obviously be expected if the antitoxin-nitrogen were not coagulated during heating

It was noteworthy that the presence of sodium chloride increased the tendency to coagulation of floccules on heating, and no samples heated under these conditions dissolved completely Whether these effects are due to sodium chloride itself or to the increase in the total ionic concentration of the suspending solution has not been investigated

The effect of time of heating upon antigenic efficiency and toxoid liberation

In view of the fact that floccules heated under conditions favouring their complete solution dissolve in a very short time (10–15 minutes), it appeared possible that any changes that occur might take place very quickly, and that prolonged heating might either be unnecessary or produce a detrimental effect Experiments were therefore carried out to determine the optimum time of heating to liberate maximum amounts of toxoid, and to produce the best type of antigen Table VII shows the effect of time of heating of two batches upon the amount of toxoid passing into solution at different pH levels In some instances, e g Samples 4 and 6, time has but little effect upon toxoid liberation, and usually the maximum was reached in an hour In all experiments involving Batch Y, destruction of toxoid occurred with longer heating ; with Batch X, destruction occurred only at pH 8 5 between 1 and 2 hours Unless this were due to individual batch-variation, it appears possible that splitting of floccules made from refined

TABLE VII —*Showing the Amounts of Free Toxoid Detectable in Filtrates from Toxoid-Antitoxin Floccules after Heating for Different Lengths of Time*

Sample	Batch of floccules	Type of antitoxin in floccules	pH	Temperature	Lf per ml in filtrate after heating for—			
					15	30	60	120 min
1	X	Unconcentrated	7 05	75° C	6	12	11	13
2	X	,,	8 0	75° C	16	24	28	28
3	X	,,	8 5	75° C	45	49	72	54
4	Y*	Refined	7 05	75° C	5	5	6	4
5	Y	,,	8 0	75° C	14	13	19	9
6	Y	,,	7 05	80° C	9	10	7	7

* This batch contained only 80 Lf per ml

antitoxin occurs more rapidly than splitting of those from unconcentrated antitoxin, and that the toxoid thus liberated is somewhat more stable to heat in the latter case than the former Presumably both rate of splitting and subsequent stability are to some extent dependent upon the firmness of union of the floccule components Solubility of floccules may also be dependent upon firmness of union, for it has repeatedly been noticed in these laboratories that non-avid antitoxins (i e , those combining loosely with toxin) give a very meagre yield of floccules, suggesting that all the toxoid-antitoxin does not come out of solution It is possible that floccules from refined antitoxin are more readily soluble than those from unconcentrated antitoxin, though this has not been shown, and in their behaviour on heating at pH 8 5 or above there is no observable difference

If the antigenic efficiency. of heated preparations were related entirely to their free toxoid content, it would be expected that time of heating would have but little effect, with most of the preparations shown in Table VII That this is in general not the case is shown in Table VIII, which deals with most of the

TABLE VIII —*Showing the Immunity Response of Guinea-pigs to 2 Injections of Some of the Suspension Featured in Table VII*

Sample in Table VII	Dose injected	Median antitoxic value of guinea pigs injected with samples heated—			
		15	30	60	120 min
1	0 01	—	0 163	—	0 163
3	0 0067	0 100	0 130	0 0063	0 0040
4	0 01	0 0470	0 141	< 0 001	< 0 001
5	0 01	0 014	< 0 001	< 0 001	< 0 001
6	0 01	0 163	0 130	< 0 001	< 0 001

samples shown in Table VII The results show that the antigenic efficiency of Samples 4, 5 and 6 was severely damaged if heating was maintained for as long as 1 hour, although the free toxoid content might increase between 30 minutes and 1 hour Using Samples 1 and 3 (from the first batch of floccules) this was not so apparent, though with Sample 3 a considerable reduction in antigenic efficiency occurred between 30 minutes and 1 hour and 2 hours, despite the fact that the 1-hour sample contained a much higher titre of toxoid than the other samples No difference in efficiency was obtained in the case of Sample 1,

whether heated for half an hour or two hours It should be noted, however, that no guinea-pigs injected with these samples gave a poor response, and it is possible that the use of lower dosage might have revealed differences

It is apparent that the majority of suspensions reach their maximum antigenic efficiency after heating for 30 minutes or less   Longer heating, though this may liberate more toxoid, damages the antigenic properties, in some cases severely This effect is thought to be due to denaturation of toxoid in solution, rather than to any effect on the remaining floccules, since Sample 3 in Table VIII became completely dissolved after 10 minutes' heating, so that all the samples in this series were clear solutions

These experiments show that the amount of toxoid passing into solution on heating under given conditions of pH and temperature is dependent upon the composition of the floccules heated , that, in general, increase of pH or tempera- ture causes liberation of greater amounts of toxoid   The addition of relatively small concentrations of sodium chloride to suspending buffers may make an enormous difference to the heated product, and time of heating has a very significant, but not constant, effect upon the antigenic efficiency and toxoid content of the resulting suspension   Other experiments, not described here, have shown that batches of floccules of the same composition in terms of toxoid antitoxin ratio, but containing different toxoids, do not behave in the same manner when heated under apparently identical conditions , it is not possible to predict, *a priori*, the yield of soluble toxoid under any given conditions, nor whether coagulation will occur   This may be due to the presence of traces of certain inorganic salts in some batches   Further, relatively large amounts of antitoxic protein are present in most filtrates, and it is surprising that Watson, Taggart and Shaw describe their filtrates as " highly " concentrated and purified antigens " containing mere traces of dissolved matter " The purest filtrate obtained in my experiments contained less than 800 Lf per mg of nitrogen The experiments also suggest that some of the material present in the filtrates exists as loosely-combined undestroyed antitoxin , this has actually been demonstrated by means of the guinea-pig intracutaneous test   The addition of toxin to a filtrate has turned out of combination some of the toxoid, thereby sensibly reducing the toxicity of the toxin through combination with traces of freed antitoxin   In these same filtrates the presence of toxoid was demonstrated by a quantitative flocculation test   The nature of the filtrates is therefore not simple, but appears to consist of toxoid, under certain conditions in a somewhat denatured state, in loose combination with antitoxin   The quantitative relation between the toxoid and antitoxin cannot be worked out by any simple means, but it is probable that the toxoid is grossly in excess   It is also possible in such a system that combination in multiple proportions may occur

SUMMARY

When diphtheria toxoid-antitoxin floccules are heated, more toxoid is recovered under the same conditions of pH and temperature from underneutralized floccules containing less than one equivalent of antitoxin than from those prepared with one equivalent or more   The amount of toxoid released decreases as the proportion of antitoxin in the floccules increases   Suspensions heated above pH 8 2 usually dissolved completely

The antigenic efficiency of floccules heated under conditions so as to recover varying amounts of toxoid increases with the amount of toxoid released, appearing to be maximal when 30–40 Lf/ml are present in solution, but decreases again at a concentration of 60 Lf/ml   The original floccules contained 120 Lf/ml of toxoid

The antigenic efficiency of unheated floccules plus heated floccules solution is inferior to that of heated floccules, suggesting that heating brings about changes in the floccules other than dissociation, and destruction of antitoxin

The presence of sodium chloride in suspending buffer inhibits liberation of toxoid on heating   This may, however, be due to the increase in the total ionic concentration

The purest filtrate obtained contained 790 Lf doses per mg nitrogen, showing that considerable quantities of soluble antitoxic protein were present   This was obtained from floccules of composition 1 Toxoid   0 67 antitoxin   purities of filtrates from 1 1 or 1 1 5 floccules were considerably lower

The antigenic efficiency of suspension of floccules increases on heating for 15 to 30 minutes, after which it is usually severely damaged, even if no destruction of soluble toxoid occurs

The results suggest that filtrates from heated floccules consist of relatively small amounts of antitoxin loosely combined with and grossly over-neutralized by toxoid

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THE LEWIS BLOOD GROUPS OF 79 FAMILIES

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THE "Lewis" blood groups were discovered in 1946 by Mourant, who found a "new" antibody in two samples of human serum. This antibody, which was called anti-Lewis, agglutinated the red blood cells of 24 out of 96 English people of group O. The antigen thus recognized appeared to be independent of the ABO, MN, Rh, P and Lutheran antigens.

The antibody gave reactions slightly stronger at room temperature than at 37° C., both examples were considered to be spontaneous rather than immune.

Mourant tested the families of 15 persons who were Lewis positive. In 7 cases both parents of a Lewis-positive person were examined, and one or the other parent was also Lewis positive in all of them. Mourant said, "This is conclusive evidence that the agglutinogen is inherited, and suggestive, but not statistically significant evidence that it is a Mendelian dominant character rather than a recessive." Considering that all other known blood group genes express themselves in single dose this restraint was most praiseworthy, and justified in the light of subsequent investigations.

More examples of anti-Lewis sera were quickly found, a description of one was published by Jakobowicz, Simmons and Bryce (1947). Several were sent to Dr. Mourant, at the Blood Group Reference Laboratory, from the English and Welsh Blood Transfusion Services. An example was found by Dr. Hartmann in Oslo, and one of us (R. S.) found 4 examples in a collection of antisera set aside for her investigation by Dr. L. K. Diamond, at the Blood Grouping Laboratory, Boston.

In 1947 Andresen reported that he and Freiesleben, working independently, had found, during the course of the year, 8 examples of an antiserum which agglutinated the red cells of 21 per cent of adults. Andresen (1947) called this blood group system the L system, which was in a way fortunate, for it proved subsequently to be the Lewis system of Mourant. Andresen's choice of a symbol was in another way less fortunate, for there was at the time in the English press a paper by Mainwaring and Pickles (1948) describing 3 examples of the anti-Lutheran antibody of Callender and Race (1946), and defining subdivisions of the Lutheran groups for which Mainwaring and Pickles used the symbols L_1, L_2 and l.

Andresen's paper considerably advanced knowledge of the Lewis blood groups. The frequency of Lewis-positive blood in adult Danes was established as 21 per cent, and the unexpected observation was made that the frequency was higher in infants. These points are illustrated in Table I, which is taken from

TABLE I —*Type Distribution Demonstrated in Adults and in Children at Different Ages (from Andresen)*

Type	Adults		0–3 months		4–6 months		7–9 months		10–12 months	
	No	%	No	%	No	%	No	%	No	%
L+ .	166	21	78	79	74	73	18	36	19	29
L— .	618	79	21	21	28	27	32	64	46	71

Andresen's paper   Andresen found, moreover, that " parents of type L— very well might get children of type L+," and in order to satisfy these two sets of observations proposed that, while in adults only LL homozygotes gave the reaction L+, in young infants on the other hand the heterozygotes Ll were also giving the L+ reaction   In other words, in adults the Lewis+ group was behaving as a recessive Mendelian character   This was unexpected, for perhaps all other known blood group genes express themselves when in single dose   One of the main purposes of this present paper is to give evidence which appears strongly to support Andresen's view that Lewis-positive adults are genetically homozygous

In 1948 Andresen published a second paper which again advanced the subject Andresen reported the finding of another type of antiserum (anti-L_2), whose reactions bore an almost completely antithetical relationship to those of the original anti-Lewis (called by Andresen in this second paper anti-L_1)   Table II, which is taken from the paper referred to, shows the result of testing 238 group O blood samples (evidently from adults) with the two sera

TABLE II —*Distribution of the Groups in the L System within Group O (from Andresen)*

	$L_1+ L +$	$L_1+ L_2—$	$L_1— L_2+$	$L_1— L_2—$	Total
Group O . .	0	46 (20%)	178 (74%)	14 (6%)	238

As Andresen pointed out, these results fit very well with the concept that L_1 and L_2 are allelomorphic antigens, but that the existence of the small $L_1—L_2—$ group made it " not yet possible to establish the manner of inheritance with certainty "

Such were the results obtained by Andresen with adult group O cells and also with group A_2 cells   When A_1 cells were tested, however, it was found that only 42 per cent were agglutinated by the anti-L_2 serum, and that many of the reactions were weak or doubtful   Andresen considered this effect to be an example of the phenomenon known in genetics as epistasis, L_2 being suppressed if A_1 were also present   Epistasis is defined by Waddington as follows " Another form of interaction between multiple factors is found when one factor is ' dominant ' over the others, i e suppresses their expression   We cannot use the terms dominance and recessiveness in such cases since the factors involved occupy different loci and are not allelomorphs   Bateson proposed the words epistasis and hypostasis "   Andresen found that in children the percentage of group O bloods agglutinated by the anti-L_2 serum was considerably lower than in adults

In July, 1948, Dr Andresen visited England and kindly demonstrated the reactions of the anti-L_2 serum   Later we recognized a second example of anti-L_2 in a serum sent to us by Dr Peter Vogel and Dr R E Rosenfield of New York

In October, 1948, Grubb, working at the Lister Institute, made an observation which greatly added to the interest of these many-sided groups, and which started a fresh line of investigation   Grubb discovered that practically all, if not all, persons whose red cells were Lewis positive (L_1+) were also salivary non-secretors of A, B or H substance, and *vice versa*

Early in 1949 certain people engaged in work on the Lewis groups (Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles and Race, 1949) agreed to use the following notation for the Lewis and Lutheran systems

System	Lewis	Lutheran
Genes	Le^a	Lu^a
	Le^b	Lu^b
Genotypes	Le^aLe^a	Lu^aLu^a
	Le^aLe^b	Lu^aLu^b
	Le^bLe^b	Lu^bLu^b
Phenotypes	Le(a + b —)	Lu(a +)
	Le(a — b +)	Lu(a —)
	Le(a — b —)	
Antibodies	anti-Le^a	anti-Lu^a
	anti-Le^b	

No distinction is made between the symbols for the genes and those for the antigens

The antibody anti-Le^a corresponds to the original (Mourant, 1946) anti-Lewis and to the anti-L_1 of Andresen, anti-Le^b corresponds to the anti-L_2 of Andresen (1948)

If only one anti-serum, anti-Le^a, is used the phenotypes which it defines are called Le(a +) and Le(a —)

This notation will be used in what is to follow

<div align="center">EXPERIMENTAL</div>

Samples of blood from 571 unrelated persons have been tested with anti-Le^a, 130 or 22 77 per cent were agglutinated   Table III shows the samples divided

TABLE III —*Lewis Blood Groups of 571 Unrelated Persons*

	O	A_1	A_2	B	A_1B	A_2B	A_1 A_2 undifferentiated		Total
							A	AB	
Le(a+)	59	47	9	9	2	1	3	0	130
	21 93%	25 68%	18 37%	21 95%					22 77%
Le(a—)	210	136	40	32	6	6	10	1	441
									77 23%
Total	269	183	49	41	8	7	13	1	571

according to the A_1 A_2 B O blood groups In view of the influence of the A_1 antigen on the reaction of anti-Le^b serum the following comparison has been made

	Le(a +)	Le(a −)		Le(a +) %
Samples containing A_1 . .	49	142	.	25 65
Samples without A_1 . .	78	288	.	21 31

But as $\gamma^2 = 1\ 3$ for one degree of freedom, the excess of Le(a +) blood samples which contain A_1 can well be accounted for by chance, and it can be assumed that the reaction of a sample of blood with anti-Le^a is independent of its A_1 A_2 B O group

Of the 571 blood samples 406 were from English people , 132 were from American whites, tested in Boston by one of us (R S), and 33 were from people known to be Welsh

Although the exact manner of inheritance of the Lewis groups as defined by anti-Le^a and anti-Le^b is not yet established with certainty, the following considerations confined to the results given by the original anti-Lewis (anti-Le^a) type of serum, strongly support Andresen s theory that the presence of the Le^a antigen is in adults a recessive character

If Le(a +) blood is genetically homozygous (Le^{a}Le^a), then the gene frequency of Le$^a = \sqrt{0\ 2277} = 0\ 4772$ and that of the other allelomorphs $= 1 - 0\ 4772 = 0\ 5228$ Representing the other allelomorphs by Le$^?$ the expected genotype frequencies would then be ·

Le(a +) . .	.	Le^{a}Le^a	0 2277
Le(a −)	.	(Le^{a}Le'	0 4990
		(Le'Le'	0 2733

and these figures can be applied to the examination of the results of testing the 79 families given in Table IV

The families have been tested for other blood group systems than those shown in the table but the addition of these groups would make the table too involved for the present purpose All the children are over the age of 2 years

Table V shows the children expected from the various types of mating, on the assumption that Le(a +) blood is homozygous

From Table V it can be calculated that the children from a mating Le(a +) × Le(a −) should be in the ratio of 0 3230 Le(a +) to 0 6770 Le(a −) From the mating Le(a −) × Le(a −) the children should be in the ratio of 0 1044 Le(a +) to 0 8956 (Le(a −) From the mating Le(a +) × Le(a +) all children would be expected to be Le(a +)

Table VI shows the comparison between the children expected and those observed in the 79 families The agreement is seen to be very close , and this strongly supports the theory of Andresen

As a contrast Table VII shows the observed children from the various matings compared with the expected children, but with the expectations this time based on the obvious alternative possibility that Le(a +) blood may be either homozygous or heterozygous for Le^a The following gene frequencies have been used in the calculation of the children expected

$$\text{Le'} = \sqrt{0\ 7723} = 0\ 8788 \text{ and Le}^a = 1 - 0\ 8788 = 0\ 1212$$

TABLE IV —*The Lewis and the $A_1 A_2 B O$ Groups of 79 Families, some of them also Classified for A, B and H Secretion*

Matings Le(a −) × Le(a +)

Family No	Father $A_1 A_2 BO$	Le(a)	Mother $A_1 A_2 BO$	Le(a)	Children Le(a −)	Children Le(a +)
1	A_1	+	A_2	−	O	A_2 A_2
2	A_1 B	+	O	−	B	
3	A_1	−	O	+		O A_1
4	A_1 ss	+	A_1 S	−	A_1 A_1 S S	
5	O	−	A_1	+	A_1	A_1
6	A_1	+	O	−	A_1	A_1 A_1
7	A_1 ss	+	A_1 S	− .	A_1 S	A_1 A_1 ss ss
8	O	+	A_1	−		A_1
9	O	+ .	O	−	O O O	
10	A_1	−	A_1	+		A_1 A_1
11	O	−	A_1	+		A_1 A_1 O ss
12	A_1	+	A_1	−	A_1	A_1
13	A_1	−	A_1	+	O	A_1 O O
14	A_1	+	B	−	A_1 B B	
15	O	+	A_1 B	−	B B	
16	A_1 B ss	+	O S	−	A_1 B B A_1 A_1 B S S S S S	
17	O	+	O	−	O O	
18	O	−	O	+	O O	
19	A_1	−	B	+		A_2 A_1 B A_2 B
20	O	+	O	−	O O O	
21	A_2	−	O	+	O S	
22	A_2	+	A_1	−	A_2 A_1 S	
23	O ss	+	B	−	B	. O
24	A_1	−	A_1	+	A_1	
25	A_1 S	−	O ss	+	A_1 O S	
26	A_2	+	O	−	O S	
27	. O ss	+	O S	−	O O O S S S	O ss
28	O S	− .	O ss	+	O O S S	
29	A_1 ss	+	O S	−		A_1 ss
30	B S	−	O ss	+	O S	B O ss ss
31	O ss	+	A_2 S	−	A_2 O O S S S	

TABLE IV (continued)

Matings Le(a −) × Le(a +)

Family No	Father A₁ A₂ B O	Le(a)	Mother A₁ A₂ B O	Le(a)	Children Le(a −)	Children Le(a +)
32	A₁ (ss)	+	A₁ (S)	−	..	. A₁ (ss)
33	A₁ (ss)	+	O (S)	−	A₁ A₁ (S S)	.
34	A₂ (ss)	+	A₁ (S)	−	A₂ A₁ (S S)	A₁ (ss)
35	O (ss)	+	O (S)	−	.	. O (ss)

Matings Le(a −) × Le(a −)

Family No	Father A₁ A₂ B O	Le(a)	Mother A₁ A₂ B O	Le(a)	Children Le(a −)	Children Le(a +)
36	A₁	−	O	−	O	O O
37	B (S)	−	B (S)	−	B O (S)	
38	O (S)	− .	. O (S)	− .	O O	.
39	A₁	−	O (S)	− .	.	. O
40	. A₁	− .	. A₁ (S)	− .	O A₁ A₁ (S)	.
41	O	−	A₁	− .	.	O
42	A₁ (S)	−	O (S)	− .	O (S)	
43	B	−	O	−	O	
44	A₁	−	A₂	− .	O	
45	O	−	A₁	−		O
46	O	−	O	−	O O O	
47	O	−	A₁	− .	O A₁	
48	A₁	−	A₂ B	−	A₂ B A₁	
49	O (S)	−	O (S)	−	O O (S)	
50	O	−	B	−	B O O B B O	
51	A₁ (S)	−	A₁ (S)	−	A₁ A₁ O (S S)	
52	O	− .	A₁	−	A₁ O A₁	
53	O (S)	−	O	−	O O O	.
54	. O	−	A₁	− .	O A₁	. .
55	A₁ B (S)	− .	O (S)	− .	B (S)	
56	. O (S)	− .	O (S)	−	O O	.
57	A₁ B (S)	− .	O (S)	− .	B A₁ A₁ (S)	
58	. A₁ (S)	− .	O (S)	− .	A₁ A₁ O (S S)	A₁ (ss)

TABLE IV (*continued*)
Matings Le(a′ —) × Le(a —)

Family No	Father A_1A_2BO	Le(a)	Mother A_1A_2BO	Le(a)	Children Le(a —)	Le(a +)
59	A_1 S	—	B S	—		O ss
60	O S	—	O S	—	O O O S S	
61	O S	—	A_2 S	—	A_2 A_2 S S	
62	A_1 S	—	A_1 S	—	A_1 A_1 A_1 S S S	
63	O S	—	A_1 S	—	O S	
64	O S	—	A_2 S	—	O O A_2 S S S	
65	A_2 S	—	O S	—	A_2 A_2 S S	O ss
66	A_1 S	—	A_1 S	—	O S	
67	A_1 S	—	A_1 S	—	A_1 S	
68	O S	—	A_2 S	—	A_2 O S S	
69	O S	—	O S	—	O O O O S S S S	O ss
70	A_1 S	—	A_1 ss	—	A_1 A_1	
71	B S	—	A_1 S	—	A_1 B S	
72	A_1 B S	—	O S	—	A_1 B S S	
73	A S	—	O S	—	O S	
74	B S	—	B S	—	B	

Matings Le(a +) × Le(a +)

75	O ss	+	A_2 ss	+		A_2 A_2 ss ss
76	B	+	O	+		O
77	O ss	+	O ss	+		O O O O ss ss ss ss
78	B ss	+	A_1 ss	+		A_1 B O O ss ss ss
79	O ss	+	O ss	+		O O O ss ss ss

The fathers of Families 55 and 57 are brothers
The mother of Family 76 is the child in Family 45

S = salivary secretor of A, B or H
ss = salivary non secretor of A, B or H

TABLE V —*Showing the Expected Group Frequencies of the Children from Different Lewis Matings   The Expectations are Based on the Theory of Andresen that Le(a +) Blood is Genetically Homozygous*

	Expected frequency of mating	Expected children		
		Le^aLe^a	$Le^aLe^?$	$Le^?Le^?$
$Le^aLe^a \times Le^?Le^a$	0 0518	0 0518		
$Le^aLe^a \times Le^aLe^?$	0 2272	0 1136	0 1136	
$Le^aLe^a \times Le^?Le^?$	0 1245		0 1245	
$Le^aLe^? \times Le^aLe^?$	0 2490	0 0623	0 1245	0 0623
$Le^aLe^? \times Le^?Le^?$	0 2728		0 1364	0 1364
$Le^?Le^? \times Le^?Le^?$	0 0747		'	0 0747
	1 0000	0 2277	0 4990	0 2734

TABLE VI —*The Expected and the Observed Absolute Numbers of Children from the Three Types of Lewis Matings   The Expectations being Based on the Theory of Andresen*

Mating		Children					
Type	Number	Number obs	Le(a +)		Le(a —)		/ for 1 d f
			Exp	obs	Exp	obs	
Le(a +) × Le(a +)	5	13	13	13	0	0	
Le(a +) × Le(a —)	35	80	25 8	30	54 2	50	0 99
Le(a —) × Le(a —)	39	84	8 8	9	75 2	75	0 01

TABLE VII —*The Expected and the Observed Absolute Numbers of Children from the Three Types of Mating   The Expectations are Based on the Assumption, no longer held that Le(a +) Blood may be Genetically either Homozygous or Heterozygous*

Mating		Children					
Type	Number	Number obs	Le(a +)		Le(a —)		$/^2$ for 1 d f
			Exp	Obs	Exp	Obs	
Le(a +) × Le(a +)	5	13	10 2	13	2 8	0	
Le(a +) × Le(a —)	35	80	42 6	30	37 4	50	7 95
Le(a —) × Le(a —)	39	84	0 0	9	84 0	75	

It will be seen from Table VI that the results of testing the blood of 79 families for the two groups Le(a +) and Le(a —) are in good agreement with the contention of Andresen that adult Le(a +) blood is genetically homozygous

Table VII shows that the results contradict the alternative possibility that Le(a +) blood may be either homozygous or heterozygous for Le^a

It may be wise to avoid speaking of the Le^a antigen as behaving in adults as a Mendelian recessive character and therefore differing from other blood group antigens   Most anti-Lewis sera are of low titre, and it may be that if more powerful sera were available the Le^a gene would be recognizable in single

dose The appearance of recessivity may be due to the weakness of the antisera at present available

Samples of saliva from 86 of the parents and 86 of the children shown in Table IV have been tested for the presence of the A, B or H substances   It will be seen below that the results are in accordance with the observation of Grubb (1948) that all people so far tested whose red cells are Le(a +) are salivary non-secretors of the A B and H substances and that with a very few exceptions people whose red cells are Le(a —) are salivary secretors of the A, B and H substances

' The following figures have been counted from Table IV

		Red cells	
		Le(a +)	Le(a —)
A, B or H in saliva	Secretors	0	123
	Non-secretors	48	1

The results of the family groupings have been counted in a number of different ways in a search for relationships that might possibly exist   The most striking of these counts was the following, taken from the Le(a +) × Le(a —) matings

	Children	
	Le(a +)	Le(a —)
Mothers Le(a +)	16	11
Mothers Le(a —)	14	39

and for this 2 × 2 table $\chi^2 = 8\ 2$, which corresponds to a probability of less than 1 in 400   The main upset in the table is due to too many of the children of Le(a +) mothers being themselves Le(a +)

In attempting to decide whether this disturbance has some cause other than chance it must be remembered that the data provided by the families have been counted in about 20 different ways, and this must lower somewhat the level of probability which is to be considered statistically significant

Subdivisions of the count suggest that the disturbance, low as the probability is of it being a chance one, nevertheless may be due to chance   If the first 16 families and the last 19 families are counted separately the total number of children in each count is about the same, but the association of Le(a +) mothers and Le(a +) children is found only in the first count

First 16 Families

	Children	
	Le(a +)	Le(a —)
Mothers Le(a +)	11	2
,, Le(a —)	8	20

Last 19 Families

	Children	
	Le(a —)	Le(a —)
Mothers Le(a +)	5	9
,, Le(a —)	6	19

Had there been a real association between the Le(a) mothers and Le(a —) children the disturbance would have been expected to show in both counts

Further, if a count is made omitting all the families with only Le(a −) children, as possibly having a homozygous negative parent, the following figures are obtained

	Children	
	Le(a +)	Le(a −)
Mothers Le(a +)	16	3
,, Le(a −)	14	10

As $\chi^2 = 3\ 4$ for one degree of freedom this is a further indication that the disturbance originally observed may be due to chance

Two other counts which showed a deviation from the expected proportion will be given in order that attention may be drawn to the deviations and future work show whether they are of significance

Mating Le(a +) × Le(a −)

	Children	
	Le(a +)	Le(a −)
Mothers A_1 or A_1 B	15	13
,, O, A_2, B or A_2 B	15	37

$$\chi^2 \text{ for } 1\ d\ f = 4\ 8$$

Mating Le(a +) × Le(a −)

	Children	
	Le(a +)	Le(a −)
Children A_1 and A_1 B	17	16
, O, A_2, B or A_2 B .	13	34

$$\chi^2 \text{ for } 1\ d\ f = 4\ 7$$

Another count seems worth recording in view of the influence of the A_1 A_2 B O groups on the reactions of the anti-Le^b serum it shows no disturbance of proportion

Mating Le(a +)   Le(a −)

	Children			
	Le(a +) A_1 A B O		Le(a −) A_1 A_2 B O	
	Compatible	Incompatible	Compatible	Incompatible
Mothers Le(a −)	11	3	28	11
Le(a +)	11	5	10	1

This can be reduced to the 2 × 2 table

Mother child	Children A_1 A B O	
	Compatible	Incompatible
Lewis groups same	39	16
different	21	4

for which $\chi^2 = 1\ 6$ for 1 degree of freedom, indicating that no disturbance has been detected In this count compatible children are those whose ABO group

would permit them to give a transfusion to their mother with the qualification that an A_1 child has been scored as incompatible if the mother is A_2

More than half of the 79 families described in this paper were also tested with anti-Le^b serum   Of the blood samples tested those belonging to group O, A_2, B or A_2 B were, with four exceptions, agglutinated either by anti-Le^a or by anti-Le^b, but not by both   The four exceptions, all from unrelated persons, gave unequivocally negative reactions with both antisera   More families are being tested in the hope of throwing further light on the genes involved in the Lewis groups

SUMMARY

The Lewis blood groups of 571 unrelated persons have been determined 130 or 22 77 per cent were Le(a +)   The Lewis blood groups of 79 families are given in detail, and on analysis the results are found to support strongly the contention of Andresen that adults whose red cells are Le(a +) are genetically homozygous

The saliva of 172 members of these families has been tested for the presence of the A, B or H substances   The results entirely conform with the observation of Grubb that there is a very close, if not absolute, association between Le(a +) red cells and non-secretory saliva, and that the converse relationship, between Le(a −) red cells and secretory saliva is very close, though not absolute

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SURVEY OF PAPERS

SMITHBURN finds that the neurotropic properties of Rift valley fever virus in mice can be enhanced by serial brain passage   Intraperitoneal inoculation in mice of the adapted neurotropic virus confers increased resistance to intracerebral inoculation of neurotropic virus and complete immunity to pantropic virus (p 1)

MALCOLM, GRIESBACH, BIELSCHOWSKY AND HALL find that prolonged administration of thiourea or related compounds in the rat leads to enlargement of the parathyroids even when enlargement of the thyroid is prevented by the simultaneous administration of thyroxine   A generalized osteitis fibrosa is found in association with the enlargement of the parathyroids (p 17)

PURVES AND GRIESBACH estimate the amount of thyrotropic hormone in human serum by measuring its effect on the height of the cells of the thyroid acini in guinea pigs   Thyrotropic hormone is present in the serum of cases of treated thyrotoxicosis and untreated myxoedema, but not in cases of untreated thyrotoxicosis   In malignant exophthalmos there are two groups of cases, one with thyrotropic activity and the other with none (p 23)

O'CONNOR finds that certain acridine compounds affect mitosis in isolated chick embryo brain at lower concentrations than those necessary to inhibit respiration, and concludes that the effects on mitosis and on respiration are independent (p 30)

SMITHBURN, HADDOW AND LUMSDEN find that Rift Valley fever virus can be transmitted by mosquitoes of the *Eretmapodites chrysogaster* group (p 35)

WILSON SMITH AND WESTWOOD have investigated the validity of the influenza virus haemagglutination reaction for determining the titre of antibodies in sera   Heat killed virus is much more likely to be stable and standardizable than living virus, but can be used for antibody assay only in the absence of the non specific inhibitory substance present in all sera   The removal of the inhibitor by cholera enzyme may facilitate the accurate assay of influenza antibody (p 48)

BARR finds that when diphtheria toxoid antitoxin floccules are heated, more toxoid is recovered from floccules containing less than one equivalent of antitoxin than from those with one equivalent or more (p 61)

RACE, SANGER, LAWLER AND BERTINSHAW have determined the Lewis blood group of 571 unrelated persons   Analysis of the Lewis blood groups of 79 families supports the view that adults whose blood cells are Lewis positive are genetically homozygous (p 73)

OVOMUCIN, A SUBSTRATE FOR THE ENZYME OF INFLUENZA VIRUS I OVOMUCIN AS AN INHIBITOR OF HAEMAGGLUTINATION BY HEATED LEE VIRUS

A GOTTSCHALK AND PATRICIA E LIND

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IT was previously shown by Hirst (1942) (1) that influenza viruses are adsorbed to chicken red blood cells and elute spontaneously after a period of time, the process of elution having a temperature coefficient of about 3 over the range 27–37° C , (2) that the virus after its spontaneous elution from the cell surface is functionally intact, whereas the red cell is irreversibly changed so as to be no longer available for virus adsorption   In interpreting his exacting observations Hirst compared the interaction between the virus agglutinin and the receptor substance at the surface of the red cells with an enzyme-substrate interaction, the enzyme possessed by the virus changing the properties of the cell receptor substance

In a series of investigations Burnet and co-workers (Burnet, McCrea and Stone, 1946 , Stone, 1947 , Anderson, 1948 , Burnet, 1948a , Burnet, 1948b) have advanced much evidence to substantiate Hirst's suggestion of the presence at the virus surface of a true catalyst   The method used by Burnet to test for virus enzyme activity is based on the findings (1) that influenza virus type B, when heated at 55° C for 30 minutes, fully retains its haemagglutinating properties, but loses its ability to elute spontaneously (Briody, 1948) , (2) that certain substances are able to inhibit haemagglutination by heated virus, when mixed together with the virus prior to the addition of the red blood cells   The first observation relevant to (2) was made by Francis (1947), who showed the power of normal human serum to inhibit haemagglutination by heated influenza B virus   McCrea (1948) proved the inhibitor present in serum to be a component of the heat-stable mucoprotein fraction of human serum and prepared (McCrea, 1949) from ovarian cyst fluid a purified mucopolysaccharide highly active as "virus haemagglutinin inhibitor," i e a substance able to combine with and to fix heated influenza B virus, thereby inhibiting the agglutination of added red blood cells   Burnet (1948a) demonstrated the inhibitory power of purified blood group O substance, a mucopolysaccharide prepared from human ovarian cysts

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by Morgan (King and Morgan, 1944, Morgan 1947, Aminoff and Morgan 1948) and de Burgh, Yu, Howe and Bovarnick (1948) provided evidence for the polysaccharide nature of a very efficient inhibitor which can be extracted from human red cells and may be identical with the receptor substance of erythrocytes (Hirst 1948)

The important fact disclosed by Burnet and co-workers (Anderson 1948 Burnet 1948a 1948b) is the loss by previous treatment with active (unheated) influenza virus of the ability of the various mucoproteins and mucopolysaccharides to act as virus haemagglutinin inhibitor   Whatever may be the forces of attraction between the surface of the heat-inactivated virus and the mucoid molecule the lack of association after treatment with active virus scarcely allows of another interpretation than of a structural change in the substrate   This finding, taken together with the further observation that a soluble enzyme of *Vibrio cholerae* referred to as receptor destroying enzyme or RDE (Burnet and Stone 1947) closely imitates active influenza virus in its ability to reduce the power of mucoids to inhibit haemagglutination by heated virus (Anderson 1948 Burnet 1948a) strongly suggests the enzymatic nature of the process

At this stage it seemed desirable to investigate the chemical change involved in the interaction between active influenza virus and suitable mucoproteins   It is the aim of the experimental work to be described in a series of papers to present information regarding the composition of the product of this interaction thus affording conclusive evidence for the enzymatic character of the virus activity under discussion and obtaining some insight into the chemistry of the underlying reaction   For such an investigation it was essential to provide a virus inhibitor easily available and readily acted upon by the virus concerned   In the search for such a substance use was made of the observation by Burnet (unpublished) that hen egg-white is an efficient virus haemagglutinin inhibitor   a property rapidly lost by pre-treatment with active virus or with RDE   Lanni and Beard (1948) have also reported a similar inhibitory effect of egg-white on haemagglutination by heated swine influenza virus   In this paper it will be shown that of the various egg-white proteins the ovomucin fraction is mainly responsible for these phenomena   Since the preparation of this fraction presents no difficulty ovomucin fulfils the specified requirements

MATERIALS

Preparation of ovomucin

Ovomucin was prepared following mainly the procedure described by Young (1937)   600 ml egg-white obtained from about 20 fresh white Leghorn eggs and freed of the chalazae were chilled and poured into 1600 ml distilled water at 0°C   The precipitate was centrifuged off and washed three times with cold 0 5 per cent NaCl solution in the centrifuge   The washed precipitate was dispersed in the minimal volume of 10 per cent NaCl solution and reprecipitated by adding the dispersion to about twenty volumes of distilled water at 0° C   After centrifugation the snow-white precipitate was washed with water until washings were chlorine free and practically devoid of protein   The final product was dried over conc H_2SO_4 in vacuo   This preparation of a white, silky appearance was analyzed for moisture ash nitrogen hexosamine and reducing substances with the following result

TABLE I.—*Analysis of the Ovomucin Fraction of Egg-white*

	Moisture mg	Ash mg	Nitrogen mg	Reducing substance (as glucose) mg	Hexosamine mg
100 mg ovomucin contain	6 3	1 64	13 20	15 4 (15 1*)	11·8

* Hydrolysed with 2N HCl at 100° C for 5 hours

The figures in Table I are in fair agreement with the data given by Young (1937)   Ovomucin, once dried, even if drying is gently effected from the frozen state, is scarcely soluble in water or in NaCl solutions of various concentration It was found that the capacity of ovomucin to inhibit haemagglutination by heated virus and the loss of this capacity by pre-treatment with active virus was changed neither quantitatively nor qualitatively, if the precipitates were washed only once   Therefore, for experiments concerned mainly with the kinetics of the virus-substrate interaction the second precipitate, after one washing, was redispersed in an appropriate volume of NaCl solution to give a final ovomucin concentration of 0 5 to 1 0 per cent and a final salt concentration of 5 per cent This dispersion, showing a considerable viscosity and a faint opalescence, was dialysed in a cellophane tube for 48 hours at 4° C against two changes of 1 8 and 1 0 per cent NaCl solution respectively

It must be pointed out, however, that ovomucin, prepared from egg-white by precipitation with water, represents a protein fraction rather than an individual protein   As was shown in this Institute by Mr E L French, all ovomucin preparations contain lysozyme in various concentration, up to 15 per cent of the total ovomucin fraction   The lysozyme content of this fraction may be considerably decreased by shifting the pH of the final dispersion (in 5 per cent NaCl) to 9 7 with 1 N KOH, chilling and seeding the liquid with crystals of isoelectric lysozyme   Under these conditions lysozyme crystallizes out from the dispersion within 2 or 3 days at 4° C and is removed together with some gelatinous matter by centrifugation   By this method, described for egg-white by Alderton and Fevold (1946,) the lysozyme content of the ovomucin fraction can be reduced to about 4 per cent of the total   The dispersion, after readjustment of the pH to 7 0, is then dialysed, as described above   This procedure yields a rather uniform dispersion of the ovomucin fraction, which may be diluted with 0 85 per cent NaCl solution, if so required

It may be mentioned that separation or concentration of the virus haemagglutinin inhibitor component of the ovomucin fraction by chloroform extraction (Sevag's (1934) method as modified by Bay, Henry and Stacey (1946)) is not feasible   If an ovomucin dispersion (inhibitory titre 6400) was shaken for 30 minutes with 1/5 of its volume of chloroform and 1/50 of its volume of n-butanol and then centrifuged for 5 minutes, only 5 per cent of the inhibitor was recovered in the upper layer (inhibitory titre 300)   Submitting the upper layer to the same treatment further reduced the inhibitory power (inhibitory titre <50)

Preparation of ovomucoid, egg albumin and lysozyme

Ovomucoid was prepared according to Mörner (1894), crystalline egg albumin according to Cole (1933) and crystalline lysozyme (isoelectric) from egg-white according to Alderton and Fevold (1946)

Purification and concentration of influenza virus

Two strains of influenza virus, Melbourne (type A) and Lee (type B) were used In each case purification and concentration were carried out in the following manner 75 ml of a centrifuged pool of allantoic fluids harvested from 13-day-old chick embryos, inoculated with the respective virus 2 days previously, were mixed with 75 ml of 0 1 M phosphate buffer (pH = 8 2) 0 1 M calcium chloride solution was then added drop by drop with constant stirring in two portions of 6 8 ml and 4 5 ml respectively and the precipitate resulting from each addition spun down in a refrigerated centrifuge After washing with 0 1 M phosphate buffer (pH = 8 2) the precipitate was quickly dissolved in 10 ml of half-saturated ammonium citrate solution, previously adjusted to pH 6 3 with citric acid, and the mixture dialysed for 60 minutes against running tap water and for 48 hours at 4° C against two changes of 4 1 0 8 per cent NaCl solution The whole procedure was performed under sterile conditions, and only preparations free from bacterial contamination were used in the experiments The technique is a modification of Salk s (1941) method, first applied in this Institute by Dr McCrea

Preparation of the receptor destroying enzyme (RDE)

A culture filtrate of a suitable strain of *Vibrio cholerae* was heated at 55° C for 30 minutes in the presence of excess calcium From this material a red cell eluate was prepared according to Burnet and Stone (1947)

METHODS

Nitrogen determinations were made by the micro-Kjeldahl method, using the apparatus of Parnas and Wagner Period of combustion was 8 hours after the addition of the catalyst (selenium dioxide)

The reducing power was determined by the Hagedorn-Jensen micromethod after hydrolysis of the substance with 1 5 N HCl at 100° C for $3\frac{1}{2}$ hours in a sealed tube

The hexosamine content of ovomucin was estimated according to Elson and Morgan (1933) Hydrolysis prior to the determination was effected by 0 5 N HCl at 100° C for 40 hours, conditions found to give maximum value The figures both for the reducing power and the hexosamine content, as tabulated, are not corrected for the unavoidable loss of carbohydrate in the course of acid hydrolysis under the conditions prevailing

Titration of inhibitory activity of ovomucin

Titrations were performed essentially according to the technique described by Burnet (1948a) Serial twofold dilutions of the inhibitor, with 0 85 per cent NaCl as diluent, were prepared in 0 25 ml volumes To each tube was added one drop of Lee infected allantoic fluid previously heated at 56° C for 30 minutes and diluted so as to contain five agglutinating doses of virus per drop After shaking the mixtures were held for 30 minutes at room temperature, when 0 25

ml of 1 per cent suspension of "sensitive" fowl red cells (Anderson, 1948) was pipetted into each tube   The cells were then allowed to settle for one hour at room temperature, and the end-point, i e 50 per cent haemagglutination, was determined by observation of the pattern of the deposited cells   The reciprocal of the inhibitor dilution showing the end-point was recorded as the inhibitory titre of the preparation   The dilutions were referred to that inhibitor concentration actually used in the experiment, unless otherwise stated

For the determination of the residual inhibitor in the presence of virus the sample was diluted 1   10 with 2 per cent NaCl solution (unless stated otherwise) and heated at 65° C for 30 minutes to destroy the virus   Controls were treated identically   Titrations were then carried out as above   The inhibitor was only slightly affected by this heat-treatment

Titration of virus haemagglutinin

Haemagglutinin titrations of virus were performed according to the method of Burnet, Beveridge, Bull and Clark (1942) with the modification that a suspension of 1 per cent fowl red cells was used

Titration of RDE activity

The activity of RDE was determined, as described by Burnet and Stone (1947)

EXPERIMENTAL

The inhibitory power of ovomucin as compared with that of the other egg-white proteins

TABLE II —*The Virus Haemagglutinin Inhibitory Titres of the Various Egg-white Proteins*

Protein	Inhibitory titre referred to 1 gm of protein (dry weight)
Ovomucin fraction	$2 4 \times 10^6$ to $4 8 \times 10^6$
Ovomucoid	1×10^3
Ovalbumin	$<2 \times 10^2$
Lysozyme	$<2 \times 10^2$

It may be seen from Table II that of the various proteins present in egg-white it is the ovomucin fraction which is endowed with the power to inhibit haemagglutination by heated influenza virus   As will be shown in a following paper this inhibitory power is a property of only a small portion of the ovomucin fraction Ovomucin, as usually prepared, is not an individual protein, but rather a protein-mucoprotein mixture   The lysozyme content of the ovomucin fraction can be reduced, as described under "materials"   Its complete removal was not attempted, since the residual lysozyme, while representing only a minor impurity, most efficiently protects ovomucin against contamination by air-borne bacteria It is due to the lysozyme content of ovomucin that larger amounts of this fraction could be prepared without taking sterile precautions and that the incubation of the final product with virus at 38° C for several hours did not require the addition of an antiseptic

Reduction of the inhibitory power of ovomucin by active virus

An approximately 1 per cent dispersion of ovomucin in 1 per cent NaCl solution, the ovomucin concentration calculated on the basis of nitrogen determination, was diluted 1 4 with 0 85 per cent NaCl solution, well mixed and the respective virus added to give a final concentration of 40 to 60 agglutinating doses per ml The mixture and an appropriate control were held in a waterbath at 37° C Samples were withdrawn at suitable intervals for determination of the residual inhibitory titre
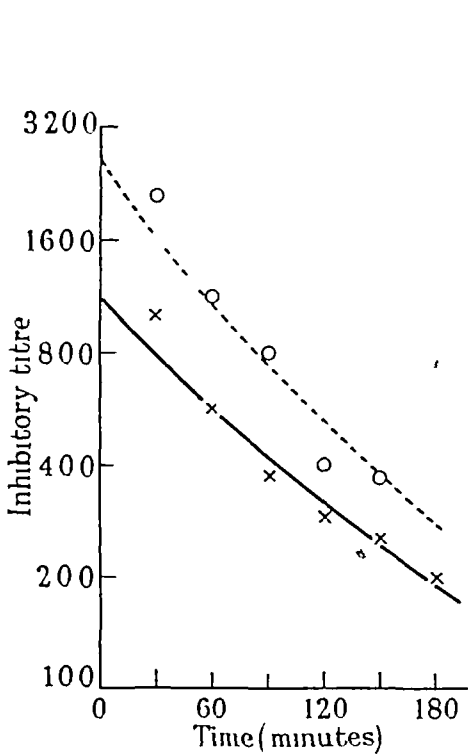
FIG 1
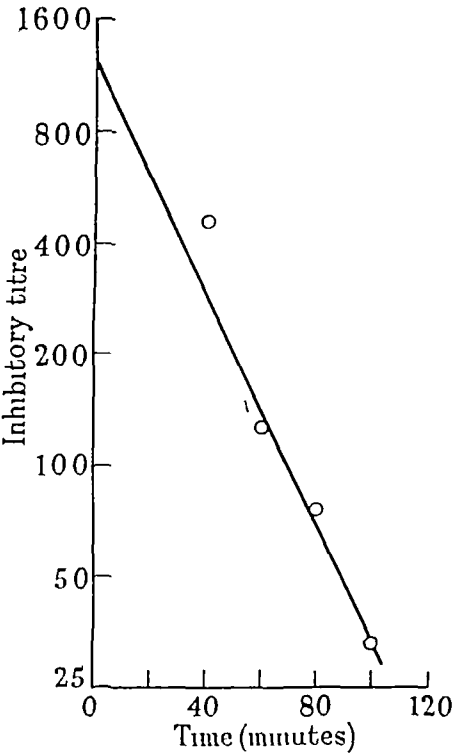
FIG 2

FIG 1 —Action of active influenza virus on the virus inhibitor of ovomucin

×————× Type A virus (Melbourne)

O - - O Type B virus (Lee)

FIG 2 —Action of the receptor destroying enzyme (RDE) on the virus inhibitor of ovomucin

The results of two experiments, one carried out with influenza type A (Melbourne), the other with type B (Lee), are shown in Fig 1 As may be seen from the slope of the lines, the inhibitory power of ovomucin is rapidly reduced by small quantities of active virus

Reduction of the inhibitory power of ovomucin by RDE

A corresponding technique, substituting RDE for virus and adding $CaCl_2$ in $M/200$ concentration, was used to investigate the effect of RDE on the inhibitory power of ovomucin The final concentration of RDE in the reaction mixture was 80 units per ml In order to determine the residual inhibitory titre of ovomucin, RDE was inactivated by preparing the serial dilutions in 0 85 per cent NaCl solution containing 0 005 M sodium hexametaphosphate as calcium deionizing agent Fig 2 illustrates the marked decrease in inhibitory titre of ovomucin by the action of RDE

Reduction of the inhibitory power of ovomucin by trypsin

The virus haemagglutinin inhibitor of the ovomucin fraction is very sensitive to trypsin action (Fig 3) The loss by tryptic digestion of the capacity of ovomucin to inhibit haemagglutination by heated Lee virus is in agreement with the finding that the inhibitory power of normal serum is also rapidly destroyed by trypsin treatment (Burnet, McCrea and Anderson, 1947, Hirst, 1948, McCrea, 1948)
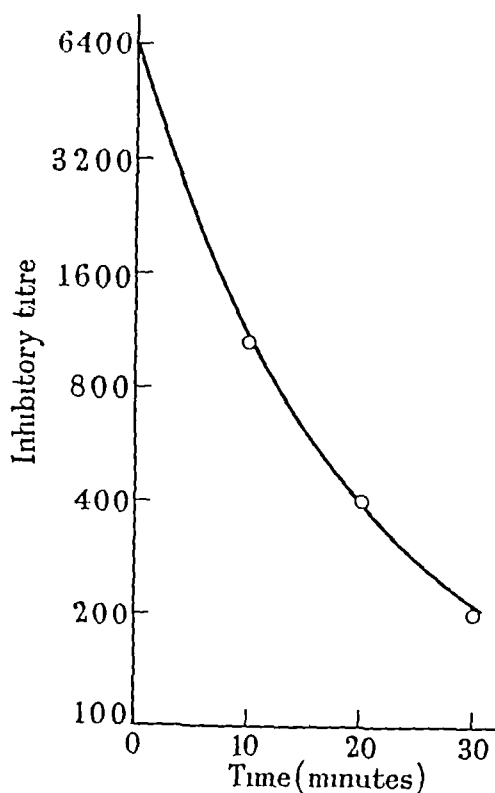

FIG 3

FIG 4

FIG 3 —Action of trypsin on the virus inhibitor of ovomucin

Conditions To 1 0 ml of 1 per cent ovomucin dispersion (in 0 85 per cent NaCl) added 1 0 ml of 0 1 M phosphate—saline buffer (pH = 7 6), containing 4 mg crystalline trypsin Temperature 37° C Samples for determination of residual inhibitory titre withdrawn at 10 min intervals
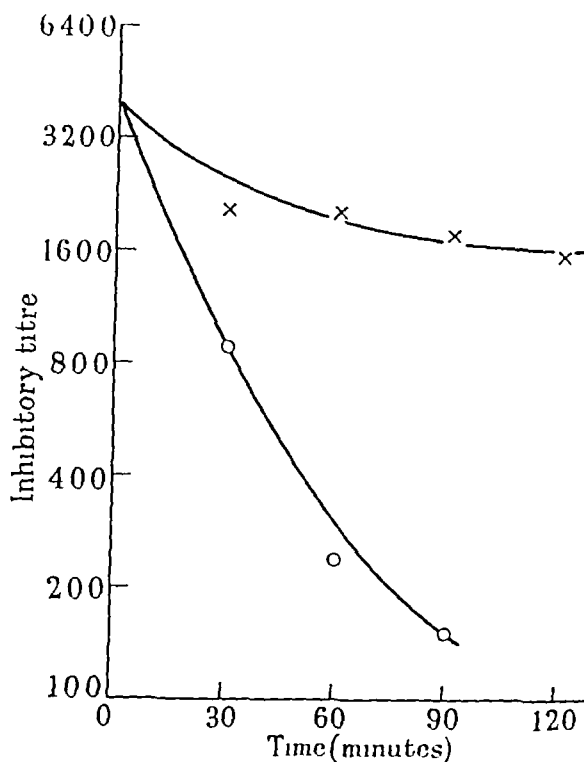
FIG 4 —Reduction by active Lee virus of the inhibitory titre of ovomucin with NaCl concentration as the variable

O————O 0 05 ml active Lee virus added to 1 95 ml of 0 4 per cent ovomucin dispersion in saline , 1·0 per cent final NaCl concentration
X————X As above, but 5 0 per cent final NaCl concentration Inactivation of virus at 65° C carried out in 5 0 per cent NaCl

Effect of NaCl on the reduction by active virus of the inhibitory power of ovomucin

Since the ovomucin is more easily and more uniformly dispersed in 5 per cent NaCl than in 1 per cent, the effect of NaCl on the virus-ovomucin interaction was investigated It is evident from Fig 4 that NaCl in 5 per cent concentration markedly inhibits the reduction by active Lee virus of the inhibitory power of ovomucin

SUMMARY

Of the various components of hen egg-white, only ovomucin has the power to inhibit haemagglutination by heated type B (Lee) influenza virus

Ovomucin, as usually prepared, is a protein fraction rather than a single protein It is always contaminated with lysozyme, in concentrations up to 15 per cent, part of which can be made to crystallize out directly from the ovomucin dispersion

The inhibitory power of the ovomucin fraction is readily reduced by active influenza virus types A and B, by the receptor destroying enzyme of *Vibrio cholerae* and also by trypsin

Sodium chloride in 5 per cent concentration inhibits markedly the reduction by active influenza virus of the inhibitory power of the ovomucin fraction

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THE APPLICATION OF PAPER PARTITION CHROMATOGRAPHY TO THE PRODUCTION OF DIPHTHERIA TOXIN, TWO-DIMENSIONAL CHROMATOGRAPHY

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IN a previous communication Linggood and Woiwod (1948) showed that when a substrain, CN 2000, of the Park Williams (P W) No 8 strain of *C diphtheriae* was grown on a weak casein hydrolysate medium designed for high titre toxin production, nearly all the cystine, aspartic acid, glutamic acid and threonine, detectable by the use of single dimensional paper partition chromatography, were utilized during toxin production, but that there was an increase in the quantity of alanine in the culture filtrate as compared with that in the original medium Although the most satisfactory separation of amino acids on the chromatogram was obtained using n-butanol acetic acid as solvent, it was not found possible to separate glycine from serine, methionine from valine and leucine from iso-leucine, but a decrease in the intensity of the spots due to these groups could be detected after the growth of the organism The results in a few instances were confirmed, using phenol and " collidine " as solvents

In order to investigate more fully the nitrogen metabolism of P W 8 and to confirm the results obtained by single dimensional chromatography, it was thought advisable to repeat the work, using two-dimensional paper chromatography to show more conclusively that, for example, the increase in the intensity of the alanine spot was, in fact, due to an increase in the quantity of this amino acid in the culture filtrate and not to the production of a polypeptide moving with a similar R_f value to alanine Two-dimensional chromatography would also reveal changes in the component amino acids of some of those groups which gave a complex spot on single dimensional chromatograms and enable us to separate glycine from serine and glutamic acid from threonine

The almost complete utilization of the glutamic acid in the medium during the growth of the organism (Linggood and Woiwod, 1948) led us to study the relationship between the amino acid metabolism and toxin production with this particular substrain of P W 8 A comparison was made, therefore of the toxin production on the basic medium, with and without added glycine or glutamic acid At the same time, single and two-dimensional chromatograms were made of such media before and after the growth of the organism but in the case of two-dimensional chromatograms of the media after growth ultra-filtrates were used in preference to the crude culture filtrates as it was considered that the presence of protein and proteose might complicate the chromatographic picture

<div align="center">METHODS</div>

Preparation of culture medium

Completely hydrolysed casein was prepared, as originally suggested to us by Dr L F Hewitt, by autoclaving Glaxo "Light White Soluble" casein (1 kg) with pure hydrochloric acid (850 ml) and water (550 ml) until the ratio of amino nitrogen, as measured by the method of Pope and Stevens (1939), to total nitrogen was maximal (70 per cent approximately)   Under our conditions, 2 hours at 121° C proved necessary   Ten such lots of hydrolysate were then bulked, diluted out to 40 litres, adjusted to pH 5 5–6 0, treated with sufficient charcoal (Sutcliffe and Speakman No 5) to give a pale coloured filtrate, which was then diluted to a total nitrogen content of 12 5–13 0 mg/ml and deferrated according to the method described by Mueller and Miller (1941)

The medium was prepared, as suggested by Holt (1948), by diluting the deferrated casein hydrolysate to a total nitrogen content of 1 0 mg/ml   Cystine, essential nutrients (pimelic acid, nicotinic acid and β-alanine) were then added as described by Mueller and Miller (1941), and the medium, after adjustment to pH 7 4–7 6, was filled out into bottles of rectangular section, 300 ml per bottle, to give a layer 1 0 cm deep   The bottles contained varying quantities of 0 1 per cent (w/v) $FeSO_4$ $7H_2O$ in 1 per cent (v/v) hydrochloric acid to give a range of added Fe   from 0 02 µg/ml to 0 20 µg/ml of medium   This medium, referred to in the text as Medium A, had a total nitrogen content of 1 0 mg/ml and a sodium chloride content of 0 45 per cent (w/v)

Toxin production on Medium A was compared with that obtained when such a medium was fortified, firstly with 0 15 per cent (w/v) of glycine (Medium B) and, secondly, with 0 29 per cent (w/v) of glutamic acid (Medium C)   Both the latter media had a total nitrogen content of 1 3 mg/ml

Bottles containing all three media were sterilized for 10 minutes at 115 5° C When cool, 9 0 ml of the maltose-$CaCl_2$ mixture, prepared as described by Mueller and Miller (1941), was then added to each bottle   This was the amount of maltose which had been found optimal for toxin production on these media under our conditions

Toxin production

The   Toronto   strain of the Park Williams No 8 strain of *C diphtheriae* was used throughout this work, the particular substrain employed being labelled CN 2000 in the Wellcome Collection   This organism was grown concurrently on Medium A Medium B and Medium C, the number of bottles being so arranged that there were at least four bottles at every level of added Fe   for all three culture media   After 8 days at 34° C the bottles were removed from the incubator and toluolised   The four bottles at each level of added Fe   were then bulked, filtered and toxin estimations done on the culture filtrates by the flocculation method (Ramon, 1922   Glenny and Okell, 1924)   With each of the three culture media   that filtrate showing the highest titre toxin was then used for paper chromatography

Ultrafiltration

For two-dimensional chromatography the culture filtrates used in this work were freed from protein by ultrafiltration through Berkefeld type candles coated

with 9 per cent (w/v) collodion in glacial acetic acid (I C I) The liquid passing through such membranes was shown to be free from protein by the addition of one-tenth volume of 50 per cent (w/v) trichloroacetic acid and also to contain no toxin as measured by the flocculation test after blending with a quick flocculating toxin (Glenny Pope, Waddington and Wallace, 1926)

Paper partition chromatography

Single and two-dimensional paper chromatograms were made of culture media, culture filtrates and ultrafiltrates of the latter, No 4 Whatman paper being used throughout For single dimensional chromatography n-butanol-acetic acid was used as solvent (Partridge, 1948), for two-dimensional work the same solvent was used initially and then phenol as solvent for running in a direction at right angles to the first This combination of solvents did not allow us to separate leucine from iso-leucine or valine from methionine, but gave a good separation of those amino acids which single dimensional chromatography had shown to be of most interest

It was found that the addition of 0 1 per cent (v/v) " collidine " to the solution of ninhydrin in chloroform (0 1 per cent (w/v)) used for spraying the papers gave a better differentiation of colour with the spots of amino acids With such a reagent glycine gave a reddish brown and serine a blue colour and so enabled us to detect changes in the amounts of these two amino acids even on single dimensional chromatograms, on which they both moved with nearly the same R_f value

All the two-dimensional chromatograms shown in this paper were run, as far as possible, under identical conditions in a constant temperature room and in many instances chromatograms which were to be compared were run in the same tank at the same time A constant volume containing approximately 50μg of nitrogen on a spot 1/5 inch in diameter was used for each two-dimensional chromatogram

RESULTS

In Fig 2 is given the photograph of the most important part of the two-dimensional chromatogram of the basic medium, i e the casein hydrolysate diluted out to a total nitrogen figure of 1 0 mg /ml with cystine and the essential nutrients added, but containing no added glycine or glutamic acid (Medium A) In the photograph each spot has been labelled with the name of the amino acid which it represented, the position taken up by each amino acid having been previously determined by running under similar conditions a mixture of pure amino acids It can be seen from this photograph that the strongest spot was due to glutamic acid, as would be expected from the fact that casein contains more glutamic acid than any other amino acid (Block and Bolling, 1945) Likewise, serine and the basic amino acids gave intense spots In contrast, the alanine present gave quite a weak spot and glycine, of which casein contains only 0 5 per cent (Block and Bolling, 1945), was just visible The behaviour of aspartic acid on our two-dimensional chromatograms was rather abnormal for it gave two spots close together As both these spots became more intense when aspartic acid was added to the medium (Fig 8) it was concluded that, under our conditions, for reasons unknown, aspartic acid ran as two spots Similar behaviour with aspartic acid on two-dimensional chromatograms has also been observed by Dent (personal communication)

Fig 4 gives the photograph of the most important part of the chromatogram of the basic medium with added glycine (Medium B), Fig 6 that of the basic medium with added glutamic acid (Medium C), Fig 8 that of the basic medium with both glutamic and aspartic acids added

Before preparing two-dimensional chromatograms of the media after growth, those culture filtrates showing the highest titre toxin on all three media were run at one time on a single sheet of paper using n-butanol-acetic acid as solvent, each filtrate in parallel with its corresponding culture medium

A photograph of the single dimensional chromatogram thus obtained is given in Fig 1, where it will be seen that with all three culture media there had been a considerable utilization of aspartic acid and an almost complete removal of glutamic acid during the growth of the organism   An increase in the intensity of the alanine spot was evident with culture filtrates from Medium B and Medium C, but not with Medium A   The disappearance, after growth, of the glycine-serine spot, except with Medium B where glycine had been added to the medium, suggested that serine might play an important part in toxin production   Although not showing in the photograph, it was noted from the original chromatogram which had been sprayed with ninhydrin in chloroform (0 1 per cent (w/v)) containing 0 1 per cent (v/v) "collidine" that the serine had disappeared during the growth of the organism with Medium B, for there was none of the blue colour of serine on the glycine-serine spot on the chromatogram of the culture filtrate, but only the reddish brown colour of glycine, whereas both colours were clearly visible on the same spot of the chromatogram of Medium B   Other points of interest which had not been noticed before were the increase in the intensities of the tyrosine and phenylalanine spots after the growth of the organism   As these observations might have been due, however, to the presence of polypeptides moving with the same R_f values as tyrosine and phenylalanine these points will be investigated more fully later

To ensure, however, that the interpretation of such single dimensional chromatograms was correct, two-dimensional chromatograms were then made of the ultrafiltrate from each culture filtrate   Photographs of the most important part of the chromatogram of Medium A and that of the corresponding ultrafiltrate are shown in Fig 2 and Fig 3   These photographs confirm the results obtained with this medium by single dimensional chromatography, as shown in Fig 1

Likewise, Fig 4 and Fig 5 give photographs of the chromatograms of Medium B which was the medium studied in our original paper (Linggood and Woiwod, 1948), and its corresponding ultrafiltrate, and present a similar picture to that for Medium A, except that the added glycine had not been completely utilized and that there had been an increase in the amount of alanine in the ultrafiltrate compared with that in the original culture medium   Thus the observations reported previously (Linggood and Woiwod, 1948) have been confirmed by two-dimensional chromatography

Similarly, Fig 7 gives the photograph of the ultrafiltrate of the culture filtrate from the culture medium to which additional glutamic acid had been added (Medium C) and should be compared, therefore, with Fig 6   It will be seen that the whole of this not inconsiderable amount of added glutamic acid had disappeared completely during toxin production and that there had been a complete elimination as far as could be detected by paper chromatography, of the slower

moving ammo acids in the solvents used with the exception of the basic ammo acids and alanine, the lattei showing an increase aftei giowth

The relative toxin production obtained on these three cultuie media is given in Table I   All Lf values quoted aie those obtained with the optimal amount

TABLE I —*Showing the Relative Toxin Production with CN 2000 on Casein Hydrolysate Media with and without Added Glycine oi Glutamic Acid*

Number	Medium A Lf units/ml	Medium B Lf units/ml	Medium C Lf units/ml
L 561	59	65	
L 566	60	61	89
L 570	67		100
L 572	61	61	
L 573	65	66	
L 574	66	61	93
L 576	65	72	104
L 582		82	104
L 587		75	97
L 588		80	92
L 595	70	76	96
L 596		90	112
L 602	86	89	116
L 605	62	61	96
L 607	64	80	86
L 609	82	103	114
L 611	75	92	104
L 620	70	86	106

Medium A   Casein hydrolysate medium (total nitrogen = 1 0 mg /ml)

Medium B   Casein hydrolysate medium fortified with 0 15 per cent glycine (total nitrogen = 1 3 mg /ml)

Medium C   Casein hydrolysate medium fortified with 0 29 per cent glutamic acid (total nitrogen = 1·3 mg /ml)

of Fe   added to the medium   From this table it will be seen that the lowest toxin production was with Medium A, while Medium B gave sometimes, but not invariably, appreciably higher toxin titres   With Medium C, howevei, high titre toxin (100 Lf units/ml $\pm$ 10 Lf units/ml) was obtained moie consistently than with Medium B   It was noted, too, that with our strain of P W 8 the growth of the organism was usually better on Medium C than on Medium A oi on Medium B, and since this work was done, bulk batches of toxin have been produced using Medium C having titres from 85–110 Lf units/ml   It was found that the pH of the culture during growth was maintained nearer to pH 7 0–7 5 with Medium C than with either Medium A oi B, both the latter having a pH at the end of growth usually between pH 6 0–6 5   When, however, less maltose was added to either Medium A or Medium B in an attempt to obtain a highei pH during growth, lower titre toxins were produced

DISCUSSION

The results obtained indicate quite clearly the value of paper partition chromatography in a study of diphtheria toxin production on weak casein hydiolvsate

medium, for not only has this technique served the practical purpose of increasing the toxin production with our strain of P W 8, but it is felt that the results are of considerable theoretical interest, pointing, among other things, to the importance of glutamic acid in the nitrogen metabolism of C diphtheriae as has already been demonstrated for other organisms (Gale, 1948)

It is appreciated that small differences of the order of 5 per cent in the quantity of any amino acid on a single dimensional chromatogram cannot be detected by inspection, but it has been shown by Woiwod (unpublished observations) that with glycine and glutamic acid differences of 20 per cent are quite obvious by inspection, especially when the materials under examination are run side by side on the same sheet of paper

It is felt, therefore, that any difference in the chromatogram of the medium before and after the growth of the organism does mean that an appreciable change has occurred in the amino acid content of the medium during toxin production On the other hand, because no difference can be detected in the intensity of the spot of a certain amino acid on the chromatogram of the medium before and after growth, it does not mean that that amino acid has not been utilized at all during growth, but only that the change, if any, taking place is not sufficient to be seen by inspection of the chromatogram It is intended, at a later date, to obtain, by the method developed by Woiwod (1948a, 1948b) for the micro estimation of amino nitrogen, quantitative figures for the changes which occur in the content of various amino acids, separated from the medium by paper chromatography, before and after toxin production

Nitrogen estimations on the washed organisms, at the end of the growth period on Medium C, at the non optimum for toxin production, gave a figure of 100 mg of bacterial nitrogen per culture bottle of 300 ml of medium The nitrogen content of the uninoculated medium was 1 3 mg /ml , i e 390 mg per bottle Hence the nitrogen content of the washed organisms represents 26 per cent approximately of the nitrogen content of the original medium Similarly, estimations of the protein nitrogen content of the culture filtrate gave a figure of 35 mg of protein per culture bottle, i e 9 per cent approximately of the nitrogen content of the original medium appears in the culture filtrate as protein nitrogen

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EXPLANATION OF PLATES

Fig 1 —Single dimensional chromatograms of Medium A, Medium B and Medium C run in parallel with their corresponsing culture filtrates with n butanol-acetic acid as solvent Key to amino acids in this chromatogram (b) basic amino acids, i e lysine, arginine and histidine , (c) aspartic acid , (d) glycine and serine group , (f) glutamic acid and threonine group , (h) alanine , (k) proline , (l) tyrosine , (n) valine and methionine group , (o) phenylalanine , (p) leucine and isoleucine group

Fig 2 —Two dimensional chromatogram of Medium A

Fig 3 —Two dimensional chromatogram of ultrafiltrate of culture filtrate from Medium A

Fig 4 —Two dimensional chromatogram of Medium B

Fig 5 —Two dimensional chromatogram of ultrafiltrate of culture filtrate from Medium B

Fig 6 —Two dimensional chromatogram of Medium C

Fig 7 —Two dimensional chromatogram of ultrafiltrate of culture filtrate from Medium C

Fig 8 —Two dimensional chromatogram of basic medium with both glutamic and aspartic acids added

Fig 9 —Complete two dimensional chromatogram of hydrolysate of washed organisms of P W 8

Fig 10 —Complete two dimensional chromatogram of hydrolysate of protein present in culture filtrate from Medium C (200 µg N taken for analysis instead of 50 µg N as with other two dimensional chromatograms)
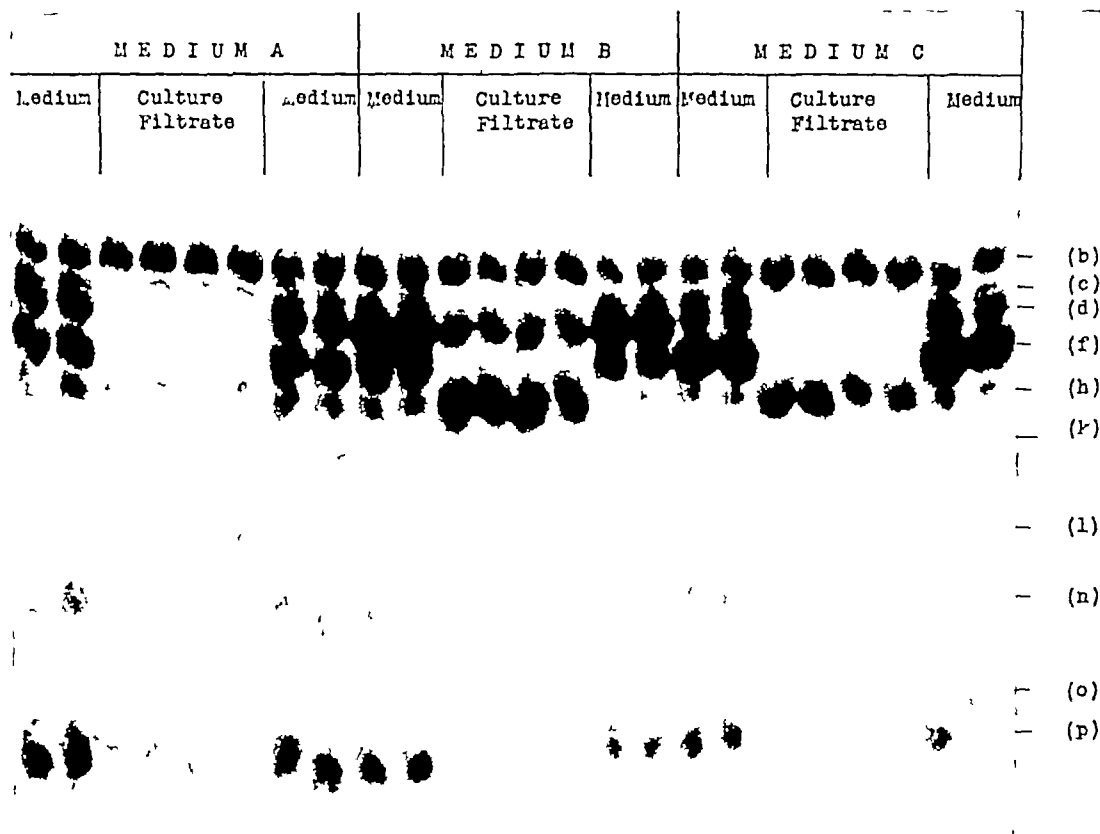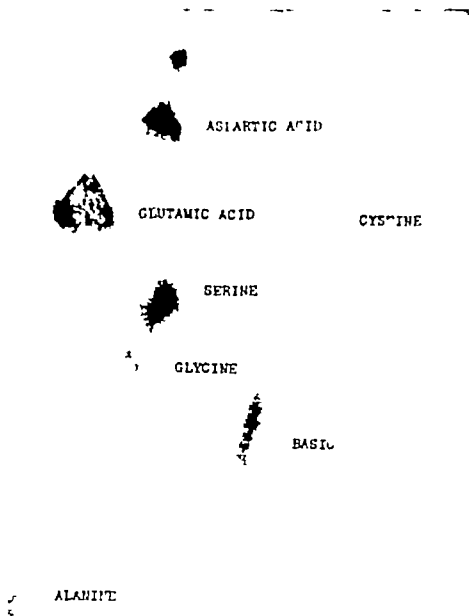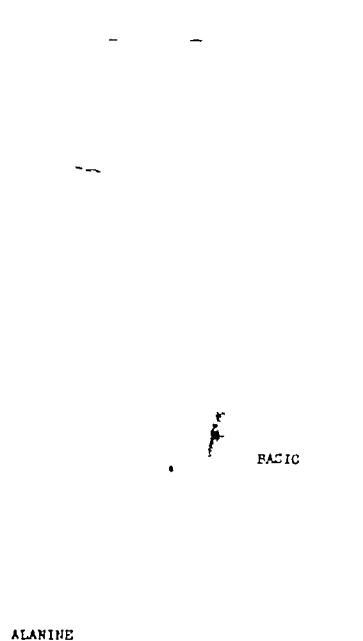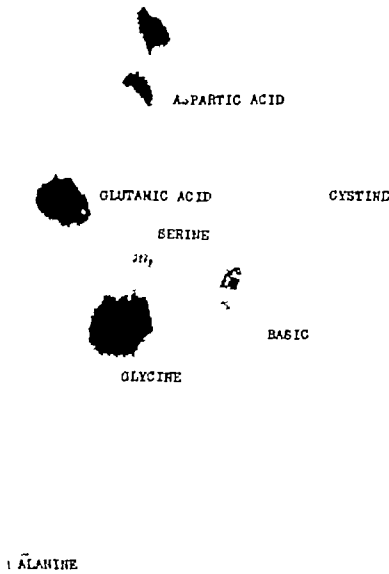
FIG 1

ASIARTIC ACID

GLUTAMIC ACID   CYSTINE

SERINE

GLYCINE

BASIC

ALANINE

FIG 2

BASIC

ALANINE

FIG 3

ASPARTIC ACID

GLUTAMIC ACID   CYSTINE
SERINE

BASIC

GLYCINE

ALANINE

FIG 4

ASPARTIC ACID

SERINE

LYSINE

GLYCINE   +

HISTIDINE

ALANINE

FIG 5

Linggood and Woiv od

ASPARTIC ACID

GLUTAMIC ACID

CYSTINE

SERINE

GLYCINE LYSINE

HISTIDINE

ALANINE

Fig 6

BASIC

ALANINE

Fig 7

ASPARTIC ACID

GLUTAMIC ACID CYSTINE

SERINE

GLYCINE

BASIC

ALANINE

Fig 8

ASPARTIC ACID

GLUTAMIC ACID

CYSTINE

SERINE

GLYCINE

THREONINE

ALANINE HISTIDINE

ARGININE

VALINE/METHIONINE

LEUCINE GROUP

Fig 9

ASPARTIC ACID

GLUTAMIC ACID

SERINE

GLYCINE

LYSINE

HISTIDINE

THREONINE

TYROSINE

ALANINE

ARGININE

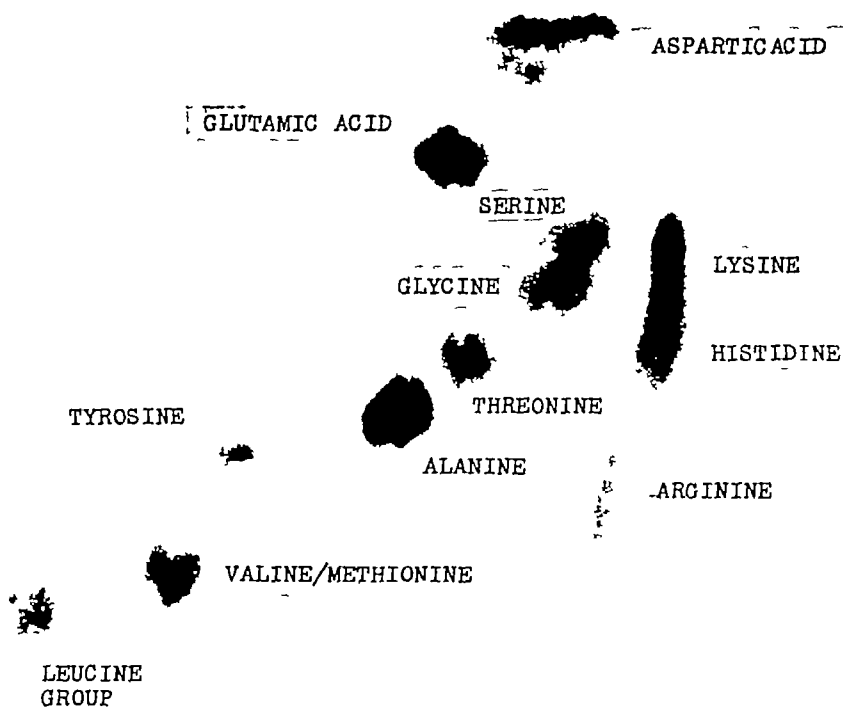VALINE/METHIONINE

LEUCINE
GROUP

FIG 10,

The nitrogen content of the added glutamic acid in Medium C is 0 3 mg /ml , i e 90 mg per culture bottle of 300 ml of medium This figure does not, however, represent the whole of the glutamic acid nitrogen in the completed medium for there is some glutamic acid in the casein hydrolysate which constitutes the basic medium From the figures given in Block and Bolling (1945) for the amino acid composition of casein, it can be calculated that in 300 ml of Medium C there is an additional 40 mg approximately of glutamic acid nitrogen due to the casein hydrolysate, making a total of 130 mg of nitrogen due to glutamic acid in one bottle of Medium C

It will be seen, therefore, if all the glutamic acid disappears from the medium during the growth of the organism, as would seem highly probable from our chromatograms, the nitrogen content of the glutamic acid which disappears is of the same order as the combined nitrogen content of the organisms and protein excreted during growth Fig 9 gives a photograph of the two-dimensional chromatogram obtained by running the hydrolysate of washed organisms and Fig 10 that by running the hydrolysate of the protein in the culture filtrate It will be seen from both these photographs that glutamic acid is by no means the major amino acid constituent present in either of these hydrolysates It is probable, therefore, that much of the glutamic acid which disappears from the medium during the growth of the organism is not used solely as a " building brick " in the production of protein, but is changed into other substances by the enzymes of the growing organism It is quite possible that among such enzymes is a transaminase as originally suggested by us (Linggood and Woiwod, 1948) to account for the increased quantity of alanine in the culture filtrates Experiments are now in progress to see whether it is possible to detect the presence of a transaminase in washed suspensions of the organism which is capable of converting glutamic acid into alanine in the presence of a keto acid (Darling, 1945)

SUMMARY

Single and two-dimensional paper chromatograms have been made of three casein hydrolysate culture media before and after the growth of our strain of the Park Williams No 8 strain of C diphtheriae These three culture media were designed for high titre toxin production , Medium A consisted of deferrated casein hydrolysate diluted to a total nitrogen figure of 1 0 mg /ml with cystine and essential nutrients added, while Medium B had an additional 0 15 per cent glycine and Medium C an additional 0 29 per cent glutamic acid added Toxin production was lowest with Medium A, usually somewhat better with Medium B, but Medium C gave more consistently high toxin titres of 100 Lf units/ml ± 10 Lf units/ml and showed the most marked changes in the chromatograms of the medium before and after growth, all the cystine, aspartic acid, serine and glutamic acid, detectable by paper partition chromatography being utilized during toxin production, while the amount of alanine in the culture filtrate increased as compared with that in the culture medium before growth

Similar changes were noted with Medium B except that the added glycine was not completely utilized Thus our original observations (Linggood and Woiwod, 1948) have been confirmed by two-dimensional chromatography Less marked changes were observed with Medium A

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Our thanks are due to Dr C G Pope and Dr A E Francis for criticism of the manuscript, and to Mr E A Jones and Mr E E Hitchcock for the photography

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LATEROSPORIN A AND LATEROSPORIN B, ANTIBIOTICS PRODUCED BY *B LATEROSPORUS*

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A CONSIDERABLE amount of work has now been done on antibacterial substances produced by sporing organisms of the genus *Bacillus* This work is summarized by Florey, Chain, Heatley, Jennings. Sanders, Abraham and Florey (1949)

Using the cross streak technique, Gilliver (1947, unpublished) showed that *B laterosporus* (Gibson, No 1066), when grown on the surface of a Lemco Agar plate produced a diffusible substance which inhibited the growth of a number of organisms An account is given below of two antibiotics that have been called laterosporin A and B produced by this strain of *B laterosporus*

EXPERIMENTAL

Assay and unit of activity

Activity was assayed by the cylinder plate method described by Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941) Laboratory strains of *Myco phlei*, *C xerosis* and *Staph aureus* (H strain) were used as test organisms A dry preparation of the hydrochloride of laterosporin A was adopted as an arbitrary standard—1 mg being taken to contain 10 units One unit per ml was found to produce inhibition zones of 14–18 mm under the conditions of the assay used

The curve relating the concentration of laterosporin A to the diameter of the zone of inhibition flattened considerably at concentrations above 1 unit per ml, and for all routine assays the test solutions were diluted to give values below 1 unit per ml. It was also found necessary to make a standard curve for each assay plate, and assays were carried out in duplicate against at least two of the test organisms

Medium and conditions of culture

For routine antibiotic production the organism was grown in the following medium—glucose 0 5 per cent, yeast extract (centrifuged water extract of dried autolysed yeast) contributing 0 5 per cent soluble solids, KH_2PO_4 0 05 per cent, K_2HPO_4 0 05 per cent, $(NH_4)_2SO_4$ 0 5 per cent, made up with tap water, pH6 6 The glucose and yeast extract, diluted with tap water, were autoclaved at 1 atmosphere for half an hour in 1 litre quantities in glass culture vessels, the medium having a depth of 40 mm A solution of 20 per cent $(NH_4)_2SO_4$, 2 per cent KH_2PO_4, and 2 per cent K_2HPO_4, was autoclaved separately and 25 ml added to each vessel before inoculating with 0 5 per cent of a 2-day culture of the organism in Lemco broth

The vessels were incubated for 3–4 days at 37° C The organism grew throughout the medium and formed a fairly thick, but easily dispersed pellicle which partly covered the surface Spores were seldom formed Approximately 1 unit per ml was produced The final pH of the medium was 5 8–6 0 When grown in 20 mm layers the activity per ml was slightly higher, but for the routine preparation of 50 or 100 litre batches it was easier to work with 1 litre of medium in a vessel

Before the medium described above was evolved a number of experiments were carried out in order to obtain as simple a medium as possible both for preparation and extraction purposes

Phosphates —The addition of phosphate was found to be necessary for optimal growth and activity However, 0 05 per cent KH_2PO_4 with 0 05 per cent K_2HPO_4 gave a higher activity than 0 1 per cent KH_2PO_4 with 0 1 per cent K_2HPO_4 If the phosphates were autoclaved with the yeast extract and the glucose, a heavy precipitate came down, probably due to reaction with the Ca and Mg ions in the tap water Growth and production of antibiotic was poor in this medium

Glucose —In early experiments the glucose was sterilized either by filtration or by separate autoclaving When the glucose was autoclaved with the yeast extract at pH 6 6 growth and production of antibiotic were delayed about 24 hours, but the maximum growth and production of the antibiotic were not significantly different from those in controls in which the glucose was added separately Autoclaving the yeast extract with the glucose at higher pH values (7 0 and 7 5) delayed growth and production of antibiotic still further Glucose was found to be necessary for optimal antibiotic production, but no significant difference could be detected between the potencies of the active liquors produced with 0 5 per cent and 2 per cent glucose

Nitrogen —Increasing the yeast extract to 1 per cent in the medium did not improve growth or activity The yeast extract could be replaced by 1 per cent Evan's peptone without loss of activity Stansly, Schlosser, Ananenko and Cook (1948) reported that the addition of 2 per cent $(NH_4)_2SO_4$ to a Difco yeast extract,

glucose and inorganic salts medium, greatly increased the yield of polymyxin without an increase in the growth of the organism as compared with the growth in the control medium with no $(NH_4)_2SO_4$ To determine the effect on the production of laterosporin, sterile solutions of $(NH_4)_2SO_4$ were added to the medium to give a final concentration of 0 5 per cent and 1 per cent The addition of 0 5 per cent $(NH_4)_2SO_4$ doubled the amount of antibiotic produced A slightly higher activity was given by 1 per cent than by 0 5 per cent Although there did not appear to be any significant difference in total growth, the organism grew faster in the presence of $(NH_4)_2SO_4$ and the maximum activity was reached earlier Autoclaving $(NH_4)_2SO_4$ with the yeast extract and glucose resulted in slightly lower activity

Extraction

The culture fluid containing approximately 1 unit per ml of activity was acidified to pH 2 0, and was either allowed to settle overnight or was centrifuged to remove cells and debris The pH of the supernatant liquor was raised to 6 5–7 0 and the active material adsorbed on to 0 5 per cent charcoal (Farnell, Grade 14, neutral) The charcoal was eluted by shaking vigorously with a mixture of 2 parts butanol and 3 parts $N/5$ HCl, the quantity used being 1/20th of the volume of the original culture fluid Almost all the activity eluted from the charcoal was concentrated in the butanol layer, not more than 2 units per ml remaining in the acid layer The method of elution from charcoal with a two phase system of butanol and HCl was first used successfully with Ayfivin by Arriagada, Abraham, Sharp, Savage, Sharp and Heatley (unpublished, 1949) The acid layer was discarded The laterosporin was recovered from the butanol layer by shaking with 2 volumes of ether and 1/5th volume of water The water concentrate was adjusted to pH 6 8–7 0 and filtered

The concentrate was cooled and laterosporin picrate precipitated by the addition of an excess of saturated aqueous picric acid solution The picrate was dried in a vacuum desiccator, ground to a fine powder, and converted to the hydrochloride by suspension in ethyl alcohol containing 5 per cent acid Not all the picrate went into solution , the insoluble portion was centrifuged off and resuspended in more acid alcohol This process was repeated until the colour of the alcoholic solution remained unchanged

The acid alcohol extracts were combined and treated with dry ether until no more precipitate came down The precipitate was washed in ether and dried *in vacuo* The light brown powder so formed had an activity of about 10 5 units per mg and was called laterosporin A The remaining material, which was insoluble in acid alcohol, was washed with ether and dried It was a yellow powder having an activity of about 12 6 units per mg and was called laterosporin B It could be freed from any insoluble material by dissolving in $N/100$ HCl containing 0 5 per cent NaCl and extracting with butanol The active material was precipitated from the butanol layer by the addition of ether and dried to form a cream coloured powder The activity of this material was approximately 11 6 units per mg , so did not differ significantly from the original laterosporin B

About 25–30 per cent of the active substances were usually recovered by the above process, but yields up to 50 per cent have been obtained The ratio of laterosporin A to laterosporin B varied, but the average ratio was 1 1 5

Chemical properties

No evidence is yet available of the purity of the preparations of laterosporin A or B

Laterosporin A —This appeared to be of a peptide nature   After hydrolysis with 5N HCl in a sealed tube at 110° C , a preliminary analysis by paper chromatography indicated that it contained a number of different amino acids

Laterosporin A was not destroyed by trypsin   A solution in water of 1 unit per ml lost no significant activity when heated at 100° C for half an hour between pH's 2 0 and 8 0

Moderate solubility at pH 2 0 decreased with increasing pH to 9 0, where it was only sparingly soluble   Partition experiments with equal volumes of butanol and an aqueous phase showed that in N/100 HCl only about 25 per cent of the activity entered the butanol layer, but that with 0 05 per cent NaCl present 66 per cent went into the butanol and with 0 5 per cent NaCl all the activity was extracted into the butanol layer

The substance was not extracted from water at any pH by ether, chloroform, or amyl acetate

Laterosporin A dialysed through a cellophan membrane

Laterosporin B —The few observations made indicated a very close resemblance of laterosporin A to laterosporin B   Laterosporin B was soluble in water and the activity of the hydrochloride against the standard test organisms was of a similar order to that of laterosporin A   Laterosporin B also showed similar properties with regard to partition into butanol in the presence of NaCl   Preliminary examination of the acid hydrolysed product by paper chromatography did not reveal any differences in the chromatogram compared with that of laterosporin A

Laterosporin B was, however, insoluble in absolute ethyl alcohol in contrast to laterosporin A, which was soluble

Antibacterial activity

The sensitivity of a number of organisms to laterosporin A and B was determined by a serial dilution method in broth   Solutions of the hydrochlorides were sterilized either by filtration through a sintered glass bacteriological filter or by heating in N/20 HCl at 97° C for half an hour   The solutions were diluted in two-fold series and the dilutions added to nutrient broth   Ten per cent of serum was present for the streptococcus and *C diphtheriae*   The tubes were inoculated with an overnight broth culture undiluted or diluted up to 1000 times   After overnight incubation the end-point was read as that concentration of laterosporin which completely inhibited growth   Further incubation for up to three days did not increase the inhibitory concentration more than two-fold   The results obtained are shown in Table I   No significant difference could be detected in the antibacterial activity of laterosporin A and B

Assays against *Myco tuberculosis* human type, strain P N , in Dubos medium showed partial inhibition at 1 unit per ml with both laterosporin A and B after incubation for 7 days   This test was performed by Dr A Q Wells

A decrease in the size of inoculum with *Staph aureus* (H strain) from an undiluted broth culture to a 1/1000 dilution decreased the inhibitory concentration fourfold at 16 hours and twofold at 48 hours

The addition of 50 per cent horse serum to solutions of laterosporin A and B reduced the activity to half its original value   No further loss of activity occurred after incubating the solutions for 4 hours at 37° C

TABLE I

Species of Organism	Concentration of laterosporin in units/ml which totally inhibited growth in 16 hours	
	Laterosporin A	Laterosporin B
Staphylococcus aureus (H strain)	0 01	0 01
Streptococcus pyogenes Group A	0 12	0 12
,, ,,	0 12	0 12
,, ,,	0 06	0 03
Corynebacterium diphtheriae var *gravis*	0 12	0 12
,, *xerosis*	0 005	0 003
Mycobacterium phlei	0 0008	0 0004
,, *smegmatis*	10 0	10 0
,, *tuberculosis* human type	*1 0 (partial)	*1 0 (partial)
Salmonella typhi	0 6	0 6
,, *enteritidis* (Gaertner)	1 2	2 5
Bacterium coli	0 6	0 6
Pseudomonas pyocyanea	5 0	10 0

* Grown in Dubos medium for 7 days

SUMMARY

An antibiotic produced by a strain of *B laterosporus* has been investigated A method is given for the production, extraction and partial purification of the active material   Some chemical properties are described and evidence presented for the existence of two separate though closely related substances which have been named laterosporin A and laterosporin B   Antibacterial activity has been demonstrated against several groups of organisms

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ACUTE EXPERIMENTAL CALCIUM CHLORIDE ACIDOSIS

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IN a previous publication (Govan and Parkes, 1946) it was shown that the administration of ammonium chloride to rabbits caused acidosis and ultimately death of the animals Widespread necrosis of the renal tubules occurred, suggesting that death might be due to uraemia In certain respects, however, the biochemical changes were atypical, and did not fit into the syndrome usually shown in acute experimental nephrosis In almost all cases of experimental nephrosis recorded there has been a marked reduction in the output of fluid and a retention of urea This has been reported in the case of poisoning by uranium (Frothingham, Fitz, Folin and Denis, 1931), tartrates (Underhill, Wells and Goldschmidt, 1913), oxalates (Dunn, Haworth and Jones, 1924), uric acid (Dunn and Polson, 1926), and by mercury (McNider, 1918) After administration of ammonium chloride the blood urea rose, but the highest recording was 173 mg per cent, and the urinary output of fluid and nitrogen remained high These results resemble those found by Folin, Karsner and Denis (1912) in animals poisoned by chromates and cantharidin It was suggested at the time that the relatively low blood urea values obtained after ammonium chloride might be due to the maintenance of fluid output, since Dunn and Jones (1925) have shown that intra-peritoneal injections of sodium chloride increased the fluid output and decreased the blood urea in rabbits rendered nephritic by oxalates It is interesting to note, however, that Underhill, Wells and Goldschmidt found that the urea output was nil, although fluid was being excreted by animals poisoned by tartrates

That there is some connection between acidosis and renal damage is suggested by the reports of diminished plasma CO_2 combining power after administration of proved nephritic agents, such as uranium (Goto, 1917), cantharidin, arsenic, diphtheria, chromates (Goto, 1918a, 1918b) and mercury (McNider, 1918 , Ogilvie, 1932) Goto (1918a, 1918b) found retention of chlorides and urea as well as acidosis in these cases, and the administration of sodium bicarbonate by mouth lessened the severity of the changes

It seemed worth while therefore to pursue the study of severe acute acidosis with the hope of eliciting further information regarding the cause of death of these animals and the relationship between acidosis and kidney function In the experiments to be described it was decided to use calcium chloride, since in our previous work it was impossible to eliminate the ammonium ion as a possible factor in the cause of death

METHODS

Experimental

Preliminary experimentation showed that doses of ammonium chloride and calcium chloride containing equivalent amounts of chlorine produced widely varying results It required a relatively greater dose of calcium chloride to produce changes similar to those obtained with ammonium chloride With the ammonium salt toxic changes resulting in death of the rabbit within four days were produced with a dose of 0 75 g per kg , but it required a dose in excess of 2 g per kg of calcium chloride to do this

Twelve rabbits were used in the experiment These were housed in urine cages Food consisted of moist bran and oats fed in bird-cage feeding boxes Urines were collected daily for the estimation of urea, chlorides, phosphates, ammonia and pH Routine tests for albumen were carried out The animals were maintained on this simple diet for a week, blood being withdrawn every second day for analysis

At the end of the week calcium chloride in a 10 per cent solution was administered daily by stomach-tube Two animals received 1 5 g per kg , two received 1 8 g per kg , two received 2 2 g per kg and six received 2 5 g per kg Blood and urine examinations were carried out as above

Chemical

Urinary and blood urea values were estimated by the hypobromite and urease methods respectively The usual Volhard technique was employed for chlorides Kuttner and Lichtenstein's (1930) method was used for the estimation of phosphates Ammonia levels were found by the aeration method The apparatus used was that described by Van Slyke and Cullen (1916) It consisted of three corked Pyrex boiling tubes connected in series by glass and rubber tubing so that air could be sucked through them by means of a water pump attached to Tube 3, the inlet being at Tube 1 Tube 1 contained 5 per cent sulphuric acid in order that traces of ammonia in the atmosphere could be removed as the air bubbled through the acid before reaching Tube 2 containing the blood and reagents Tube 3 contained 25 c c of 0 02 N hydrochloric acid Blood was then collected under liquid paraffin Five ml of blood were placed in Tube 2 One drop of caprylic alcohol and 4 or 5 g of potassium carbonate were added to the tube, which was corked and then shaken gently to mix the contents A slow current of air was then drawn through the tubes for 2 or 3 minutes, followed by a rapid current for 15 minutes At the end of this time the excess acid in Tube 3 was titrated with 0 02 N sodium hydroxide, and the necessary calculations made from the titration figure Blank determinations on the reagents were made on occasion to eliminate the possibility of ammonia being present as an impurity Serum calcium was determined, using the method of Kramer and Tisdall (1921) as modified by Tisdall (1923)

Histological

Formol saline was used as a routine fixative for paraffin and frozen sections Representative specimens for the demonstration of glycogen and phosphatase were fixed in alcohol Sections were stained by haematoxylin and eosin, Masson's trichrome method, Dunn's aniline blue Orange G method, Gomori's method for alkaline phosphatase, Best's carmine stain for glycogen and Sudan IV

RESULTS

During the first week prior to calcium chloride administration the animals appeared to be healthy Urinary volume was high at the beginning In six animals it fell slightly during the first two or three days of the new diet, but rose or remained steady thereafter Urea output was roughly parallel to water excretion, although the curve was always much flatter and changes in the curve were delayed The excretion of phosphate rose steeply, while chloride output fell Urinary pH fell gradually , blood analysis showed that urea, chlorides, lipoids, ammonia and pH were all within normal limits The CO_2 combining power of the blood was lower than usual, but still within normal limits This appears to be due to the absence of greens in the diet This feature was noted in our previous experiments with ammonium chloride Serum calcium and phosphorus were higher than usual, but the Ca P ratio was normal

In their reaction to calcium chloride the animals formed two distinct groups comprising (a) all animals receiving 1 5 to 1 8 g per kg plus one animal receiving 2 2 g per kg , and (b) all animals receiving 2 5 g per kg plus one animal receiving 2 2 g per kg

Group 1

Five animals—four receiving 1 5 to 1 8 g calcium chloride per kg and one receiving 2 2 g per kg

All of the animals in this group were killed on the 5th day of the experimental period During the first four days of this period the animals showed no signs of toxaemia, ate their food and appeared to be healthy in every way

During the first 24 hours the urinary volume diminished Urea excretion ran almost parallel with the output of water, but although the volume of urine was increased at the 48-hour period there was a 24-hours' delay in the rise of urea excretion Both were increased at 72 hours, but at 96 hours the urea output tended to fall although water excretion remained high The excretion of phosphates increased slightly during the first 24 hours, but fell moderately although progressively thereafter Chlorides, naturally, showed a steeply rising curve Urinary pH fell rapidly and remained round 5 Albuminuria occurred in all cases as soon as the pH fell below 6

The most marked change in blood chemistry was a fall in CO_2 combining power Prior to the experimental period the average normal CO_2 combining power was approximately 40 volumes per cent After calcium chloride the average was 13 5 volumes per cent At the same time the plasma pH was reduced slightly Urea was slightly increased , blood cholesterol was almost doubled, and chlorides increased by 25 per cent Calcium and phosphorus were slightly increased and the blood lipoids remained normal Table I shows a comparison of average values before and after administration of calcium chloride

TABLE I

	Blood urea, mg / 100 ml	CO_2 c p , vol / 100 ml.	pH	Cl , mg / 100 ml.	Serum Ca mg / 100 ml	Serum P mg / 100 ml.	Cholesterol mg / 100 ml.	Lipoids, mg / 100 ml	Ammonia mg / 100 ml
Before	42 1	40	7 19	594	12 0	3 76	43 6	0 14	0
After	57 7	13 5	6 8	742	13 3	4 25	70	0 13	0

The graph in Fig 1 shows the detailed changes in Animal 2, which is representative of the group   All of these animals were killed at the beginning of the 5th day of the experimental period by a sharp blow on the back of the neck   At post-mortem the appearances were similar in all cases   The lungs were emphysematous, but showed a few congested areas of collapse   No change could be
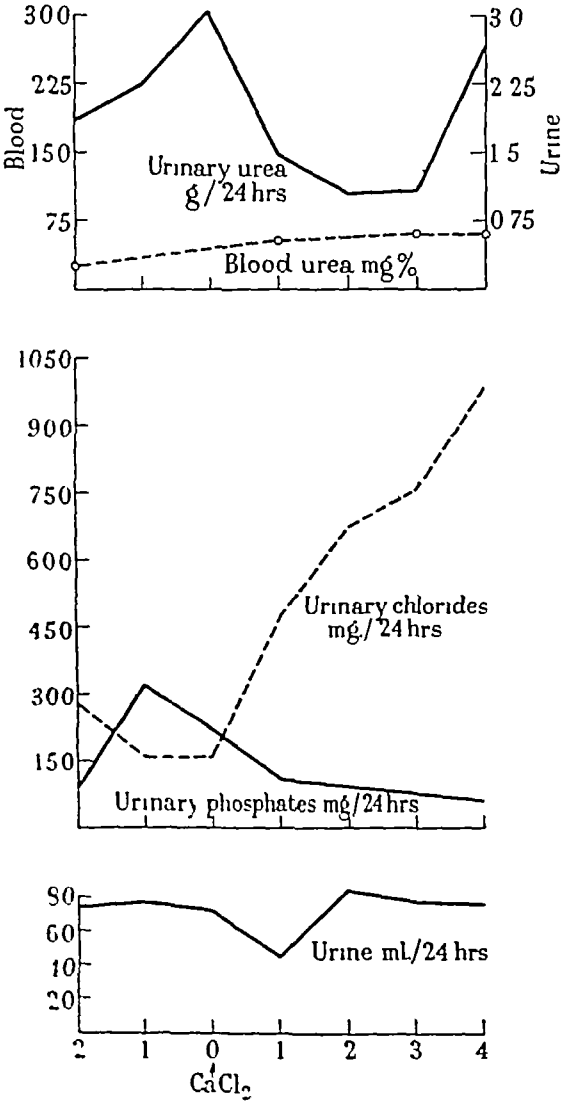

FIG 1 —Graphs illustrating the effect on Animal 2 of administering 1 5 g calcium chloride per kg for 4 days   The animal was killed on the 5th day
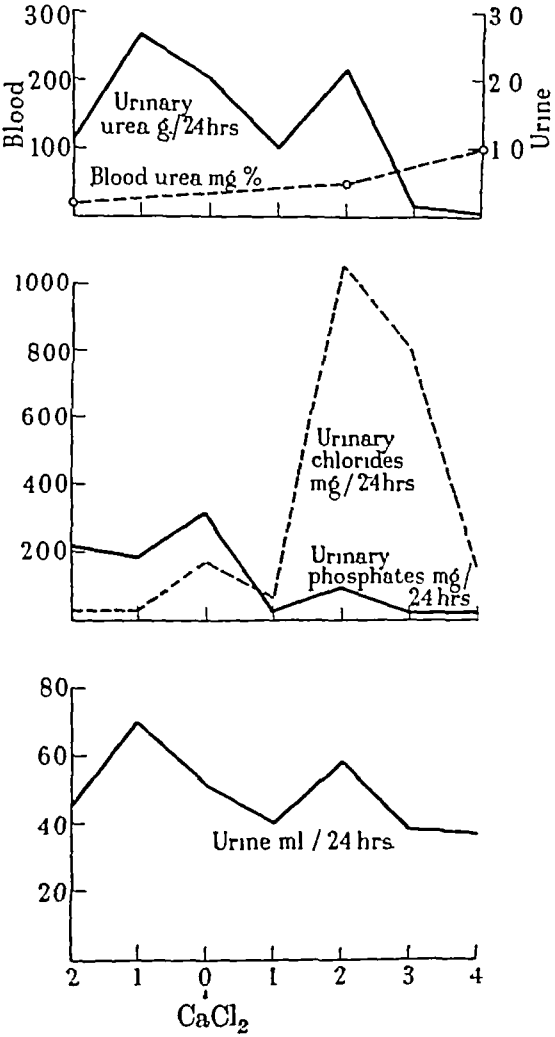
FIG 2 —Graphs illustrating the effect on Animal 8 of administering 2 5 g calcium chloride per kg for 4 days   The animal died on the 5th day

seen in the heart   The liver was slightly paler than normal, but the spleen showed no abnormality   A distinct zone of pallor was present at the junction of the renal cortex and medulla   Pieces of tissues were taken from all the organs, and fixed and stained as detailed under methods

Histology

Lung —There was marked dilatation of most of the alveoli, but occasional small areas of collapse were present   These areas of collapse were frequently in association with fat emboli, but emboli could also be seen in the emphysematous portions   The number of fat emboli varied in different parts of the lungs, but averaged approximately five per field viewed through a 2/3 in objective   A marked accumulation of polymorphonuclear leucocytes mainly of eosinphil type was present in the alveolar capillaries, and near the fat emboli these leucocytes appeared to form solid nodules   In one case haemorrhage had occurred into alveoli and bronchi, and free fat globules could be seen in the latter

Myocardium —No abnormality could be seen in ordinary paraffin sections of the myocardium   Glycogen was diminished, but most fibres still contained a small amount   Fine fatty globules were present in the myocardial fibres of one animal, but the remainder were normal

Liver —No gross abnormality could be seen in paraffin sections, but in some parts the hepatic cells contained collections of greenish-brown granules   Best's carmine stain revealed small deposits of glycogen in the cells surrounding the central veins   Fine droplets of fat were present in the Kupfer cells of the outer and mid zones, and occasionally the parenchymatous cells of the same areas showed a similar change

Kidney —The glomeruli seemed to be larger than normal and in many cases slight adhesions appeared to have developed between the tufts and Bowman's capsule   Pronounced catarrhal changes were present in the first convoluted tubules   Distal to this the nephron was dilated and the epithelium of the ascending Henle limb and second convoluted tubule was flattened

Group 2

Of 7 animals, 6 received 2 5 g calcium chloride per kg and one received 2 2 g per kg

Five of these animals died on the 5th day of the experimental period   The remaining two animals, including that receiving 2 2 g of calcium chloride per kg , were comatose on the same day and were killed   All animals exhibited similar symptoms during the last 24 hours   They became progressively drowsier, gradually losing the power of their limbs and repeatedly falling over   Finally they became comatose and died

During the first 24 hours of calcium chloride administration urinary volume diminished as in Group 1   Likewise they increased during the second 24-hour period, but by 72 hours they had begun to fall and this fall continued up to the time of death   The excretion of urea ran roughly parallel with that of water, but the rise in urea output at 48 hours did not correspond with the increase in volume   Phosphate showed a progressive fall from its previously high level Chlorides fell initially, then rose steeply, but during the last 24 hours the output fell once more   The pH, at first reduced, rose, and during the last 2 days was always above 8 0

In all cases the blood urea rose and the average was around 100 mg per 100 ml before death   A marked acidosis was present, and the CO_2 combining power of the blood was reduced to 16 volumes per cent   The pH of the blood dropped, but the fall was no greater than in Group 1   Chlorides increased as before

Serum phosphorus was doubled and serum calcium also increased   The Ca P
ratio was almost halved   Cholesterol showed a marked increase, almost 200
per cent, and plasma lipoids were augmented by almost 500 per cent   Table II
gives a comparison of the average blood values of this group with that of normals

TABLE II

	Blood urea, mg / 100 ml	CO_2 c p, vol./ 100 ml.	pH	Cl, mg / 100 ml	Serum Ca, mg / 100 ml	Serum P mg./ 100 ml	Cholesterol, mg / 100 ml	Lipoids, mg / 100 ml	Ammonia, mg / 100 ml
Normals	42 1	40	7 19	594 6	12 0	3 76	43 6	0 14	0
After $CaCl_2$	92	16	7 00	752 6	16 2	8 1	116 0	0 6	5 35

The graph in Fig 2 shows the changes in Animal 8   At post-mortem the
changes were very similar to those seen in Group 1   The depressed, congested
areas of collapse in the lung were more numerous but smaller   Marked emphy-
sema was present in the intervening lung tissue   The liver showed a generalized
pallor and the lobular markings were blurred   There was well-marked enlarge-
ment of the kidneys   The cut surface showed a distinct bulging zone of marked
pallor at the junction of cortex with medulla   The cortex also showed pallor,
but the medulla was congested   No abnormality could be seen in the other
organs

Histology

Lung —Areas of collapse were much more frequent in this group   Fat
emboli were present, but were, on the average, only slightly more frequent than
in Group 1   There was marked dilatation of the peri-vascular lymphatics, which
contained free fat globules   Nodes of eosinophil reaction were numerous (Fig
3)   A few alveolar haemorrhages were present and many of the alveoli contained
macrophages, some of which had foamy cytoplasm   In one case a slight fibrinous
exudate was present on the pleural surface adjacent to an area showing haemor-
rhage, oedema and collapse

Myocardium —Fine fatty degeneration was present in all cases   Practically
no glycogen remained, and the alkaline phosphatase reaction was negative

Liver —Glycogen was greatly diminished, but not entirely absent   A few
granules could still be seen in the cells surrounding the central vein   Focal
degenerative changes were present in the mid-zones of most lobules (Fig 4)

EXPLANATION OF PLATES

Fig 3 —Section of lung from Animal 7, Group 2, showing a solid node of eosinophils in an
     inter alveolar septum   H and E   × 340
Fig 4 —Section of liver from Animal 8, Group 2   Note the islands of necrotic cells showing
     hyperchromatic nuclei   H and E   × 85
Fig 5 —Section of liver from Animal 9, Group 2, showing areas of degeneration with karyolysis
     of nuclei   H and E   × 300
Fig 6 —Section of liver from Animal 9, Group 2, showing binucleate and trinucleate cells
     H and E   × 340
Fig 7 —Section of liver from Animal 11, Group 2, showing greenish brown granules in cytoplasm
   of cells   H and E   × 380
Fig 8 —Section of kidney from Animal 9, Group 2, showing degeneration of cells of descending
     portions of first convoluted tubules   H and E   × 340
Fig 9 —Section of kidney from Animal 8, Group 2, showing dilatation of ascending limbs of
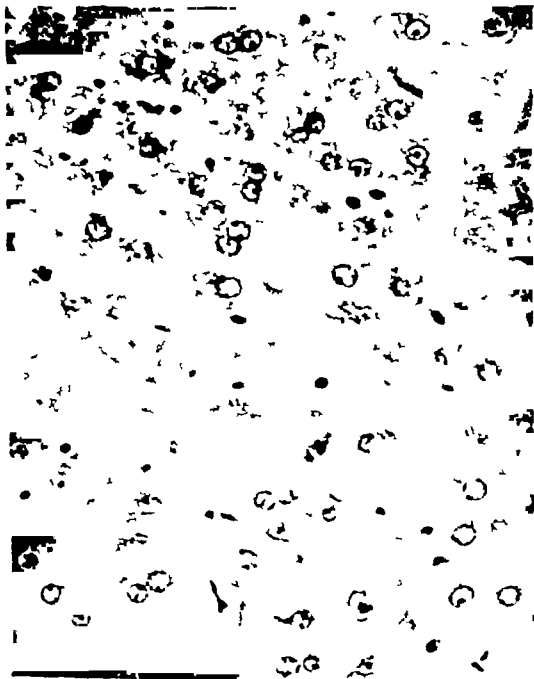   Henle   H and E   × 85
Fig 10 —Section of kidney from Animal 10, Group 2, showing pyknosis in the cells of the broad
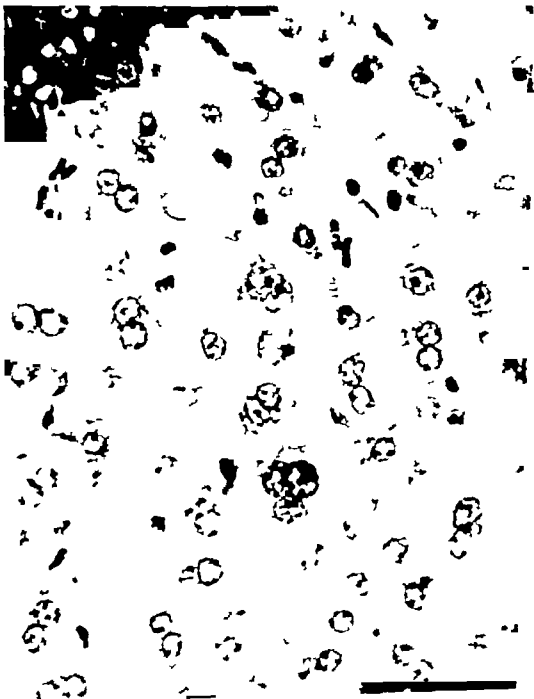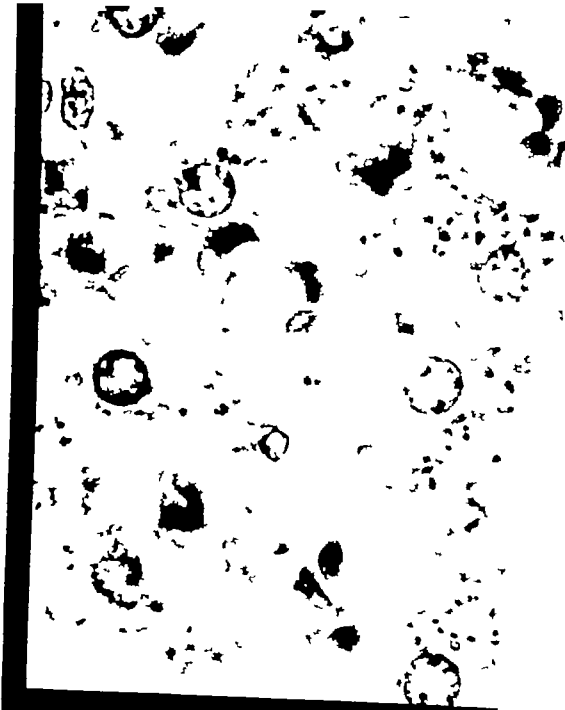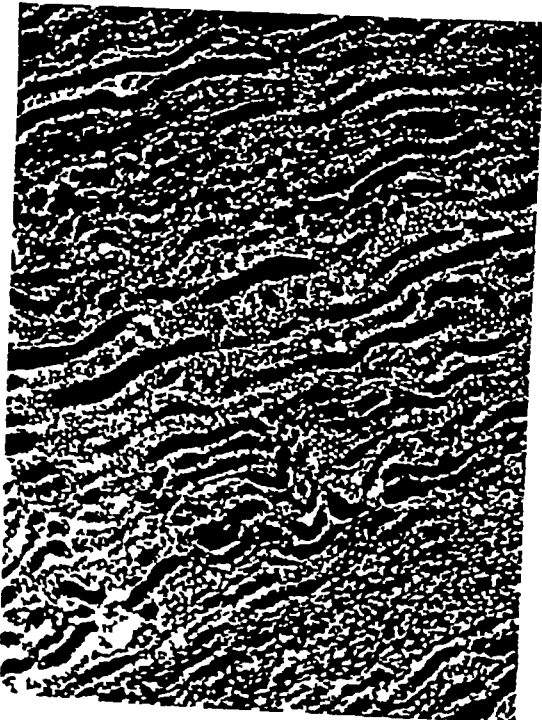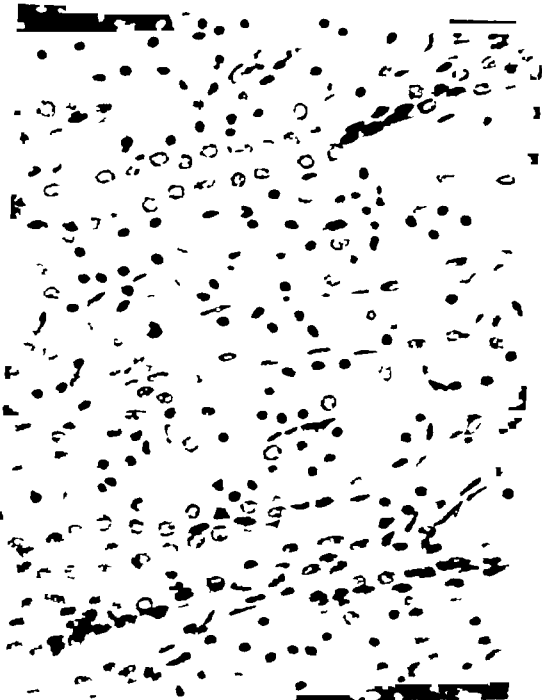   ascending limbs of Henle   H and E   × 250

3
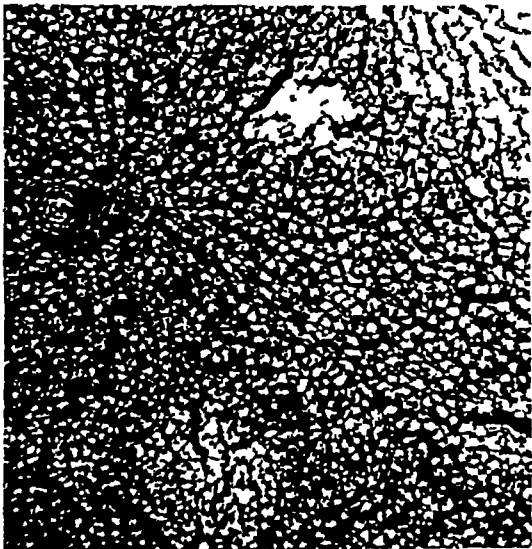
4

5

6

7

8

9

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it rose almost to 6 mg per 100 ml   The cause of this increase is not quite clear
It is possible that it represents an attempt to neutralize the acidosis, but in the
present series the blood ammonia was only increased in the second group of
animals, although the degree of acidosis as determined by the CO_2 combining
power of the plasma was no greater than in Group 1   A further proof that the
blood ammonia levels bear no relationship to acidosis can be seen in the fact that
higher levels were found in animals poisoned with ammonium chloride, although
the degree of acidosis was similar to that observed in the present series   These
findings are in agreement with those of Markert (1935, 1936)

Alternatively the blood ammonia increase may have been due to a change-
over from carbohydrate to fat metabolism, as suggested by the histological
appearance in the liver and heart   Many workers, McNeil and Levy among
them, have shown, however, that the blood ammonia is not raised in diabetic
acidosis, where it is definitely established that fat is being metabolized

The fact that ammonia appeared in the blood only in the case of animals of
Group 2 suggests that its appearance was associated with lesions found in the
organs of these animals   In this instance the most marked lesions were those
of the liver and kidneys   Ammonia is rapidly converted to urea in the normal
animal, and according the Svedberg, Maddock and Drury (1938) the sole source
of urea in the rabbit is the liver   There would therefore seem to be strong pre-
sumptive evidence in the present experiment that the increased blood ammonia
is due to liver dysfunction   Also, McNeil and Levy make a series of observations
in their paper which tends to confirm the above   These authors only found a
raised blood ammonia in acidosis if the liver was damaged   For instance, no
increase of blood ammonia could be found in nephritis unless there was a coinci-
dental myocarditis causing venous congestion of the liver   In 13 cases of acidosis,
of which 6 were diabetics, only 2 showed an increased blood ammonia   One was
a case of general peritonitis with fatty degeneration of the liver, the other a case
of persistent vomiting after laparotomy

All animals receiving 0 75 g of ammonium chloride per kg per day died after
4 days of this treatment   Calculating on the basis of chlorine content the corre-
sponding dose of calcium chloride ought to be 0 76 g per kg per day   Instead,
the dose required was 2 5 g per kg   It may be that the chlorine of the calcium
salt was not available, but the plasma CO_2-combining power was as low after
administration of 1 5 g of calcium chloride per kg as it was after 2 5 g per kg
This would suggest that the ammonium salt is more toxic than the calcium, and
this must be by virtue of the ammonium ion

It seems unlikely that the renal tubular necrosis was directly related to the
acidosis, since the changes were minimal in Group 1 and irregular in their extent
in Group 2, although all animals were profoundly acidotic   The appearance of
the kidneys at post-mortem suggests that the ultimate cause of the tubular
necrosis was anoxaemia due to a deviation of the blood from the juxta-medullary
region   The cause of this deviation is not apparent, but since the avascular
zone was present in both groups, it suggests that the altered blood flow was
directly related to the acidosis

Several minor points arise for discussion   It seems from the histological
appearances and biochemical findings that fat is mobilized from the depots long
before the glycogen stores are exhausted   Fat was present in the Kupfer cells
of the liver and in the myocardium although both organs contained small

amounts of glycogen Although the fatty change in the myocardium may be the result of degeneration, recent work (Dible, 1934 , Dible and Gerrard, 1938) suggests that the process is always one of infiltration

It will have been noted that the serum phosphorus and calcium were both raised prior to administration of calcium chloride In addition the CO_2-combining power of the plasma was reduced This latter feature was also found in our ammonium chloride series, and it appears to be related to the diet of oats and bran without greens The former changes are probably related to this mild acidosis The change in calcium concentration seems to be secondary to that of serum phosphorus This is suggested by the fact that urinary phosphorus increased during this period A further significant feature is that serum phosphorus showed a further increase during the period of calcium chloride administration These changes would appear to indicate that phosphorus plays an important role in combating the acidosis, and that probably phosphorus is mobilized from body stores

Finally, attention must again be drawn to the occurrence of pulmonary fat emboli in these experiments The meaning of this is not apparent, but it is important to realize that the reaction of the lungs to emboli is collapse—a fact which may be of some significance in cases of post-operative collapse It is also interesting to note the intense eosinophil reaction to these emboli

SUMMARY

The oral administration of 2 5 g calcium chloride per kg daily to rabbits causes death after four days

This is accompanied by extensive focal necrosis in the liver, and necrosis of tubules in the juxta-medullary region of the kidney

The results suggest that death may be due to ammonia poisoning, and that the increase in blood ammonia is related to the hepatic lesions

Following administration of calcium chloride glycogen stores are rapidly depleted, but before this is complete fat is mobilized from the depots

This mobilization results in histologically demonstrable deposits of fat in the Kupfer cells of the liver and in the myocardium, and it is accompanied by the formation of fat emboli in the lungs

The fat emboli are accompanied by a marked eosinophil reaction and cause collapse of the pulmonary alveoli

There is a retention of phosphorus after calcium chloride administration and the calcium-phosphorus ratio is diminished

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Most commonly these took the form of islands of cells staining strongly with eosin and showing pyknosis of the nuclei   These cells tended to separate from the surrounding columns   Less commonly the nuclei showed karyolysis (Fig 5), and cytoplasm, staining palely with eosin, was finely vacuolated   Signs of regeneration were present in other parts, the cells being frequently binucleate or even tri-nucleate (Fig 6)   Cells in all parts of the lobules contained greenish-brown granules (Fig 7)   Fat was present in the Kupfer cells   The phosphatase reaction was weak

Kidney —The degree of renal damage was very variable in these animals In all of them the glomerular tufts were enlarged, and in the majority light adhesions had formed between the tufts and Bowman's capsule   Albuminoid material was present in the subcapsular spaces   The capillaries were quite patent, and contained a few eosinophil leucocytes in addition to red cells   Marked catarrh was present in the first convoluted tubules and broad descending limbs of Henle   In some cases the lining epithelium was necrotic, the nuclei showing pyknosis and karyolysis   In a few cells the cytoplasm contained brown granules (Fig 8)   The distal portion of the broad ascending limbs of Henle was greatly dilated (Fig 9)   The intervening portion of the nephrons lying in the juxta-medullary region showed necrosis of many cells, mainly in the first part of the broad ascending limb   Pyknotic changes in the nuclei were marked (Fig 10) This zone was almost completely bloodless, and corresponded to the zone of pallor seen on the cut surface of the kidneys at post-mortem   Colloid casts were common in the collecting tubules   Some of the collecting tubules showed degeneration of the epithelium

DISCUSSION

In our paper on ammonium chloride acidosis (Govan and Parkes, 1946) we stated that at least two theories regarding the cause of death in these animals were possible, namely, that (*a*) the animal died of uraemia as a result of nephritis, or (*b*) they died of ammonia poisoning

Contrary to the findings in acute ammonium chloride poisoning the renal damage in the present experiment was irregular in extent and occurrence, although the symptoms exhibited and the interval of time between administration of calcium chloride and death of the animal were the same   From this alone it would seem that nephritis is not the primary cause of death in these animals, although no doubt it is a contributory factor

It has been shown that the blood ammonia appears to be greatly increased after administration of 2 5 g calcium chloride for 4 days   The possibility of spontaneous formation of ammonia from adenosine phosphate (Conway and Cook, 1939) must be taken into consideration   The original concentration may, in this way, be increased many times in a relatively short interval   Benedict and Nash (1926) and Conway (1935) showed, however, that if precautions were taken to prevent exposure to the air, and the estimations were carried out as soon as possible, then the concentration was usually less than 0 05 mg per 100 ml In the present series of experiments all possible precautions were taken to guard against this possibility, and it is significant that no appreciable amount of ammonia could be found in the rabbits' bloods prior to administration of calcium chloride   According to McNeil and Levy (1917–18) the blood ammonia in normal animals is always less than 1 mg per 100 ml   In the present experiment

it rose almost to 6 mg per 100 ml   The cause of this increase is not quite clear
It is possible that it represents an attempt to neutralize the acidosis, but in the
present series the blood ammonia was only increased in the second group of
animals, although the degree of acidosis as determined by the CO_2 combining
power of the plasma was no greater than in Group 1   A further proof that the
blood ammonia levels bear no relationship to acidosis can be seen in the fact that
higher levels were found in animals poisoned with ammonium chloride, although
the degree of acidosis was similar to that observed in the present series   These
findings are in agreement with those of Markert (1935, 1936)

Alternatively the blood ammonia increase may have been due to a change-
over from carbohydrate to fat metabolism, as suggested by the histological
appearance in the liver and heart   Many workers, McNeil and Levy among
them, have shown, however, that the blood ammonia is not raised in diabetic
acidosis, where it is definitely established that fat is being metabolized

The fact that ammonia appeared in the blood only in the case of animals of
Group 2 suggests that its appearance was associated with lesions found in the
organs of these animals   In this instance the most marked lesions were those
of the liver and kidneys   Ammonia is rapidly converted to urea in the normal
animal, and according the Svedberg, Maddock and Drury (1938) the sole source
of urea in the rabbit is the liver   There would therefore seem to be strong pre-
sumptive evidence in the present experiment that the increased blood ammonia
is due to liver dysfunction   Also, McNeil and Levy make a series of observations
in their paper which tends to confirm the above   These authors only found a
raised blood ammonia in acidosis if the liver was damaged   For instance, no
increase of blood ammonia could be found in nephritis unless there was a coinci-
dental myocarditis causing venous congestion of the liver   In 13 cases of acidosis,
of which 6 were diabetics, only 2 showed an increased blood ammonia   One was
a case of general peritonitis with fatty degeneration of the liver, the other a case
of persistent vomiting after laparotomy

All animals receiving 0 75 g of ammonium chloride per kg per day died after
4 days of this treatment   Calculating on the basis of chlorine content the corre-
sponding dose of calcium chloride ought to be 0 76 g per kg per day   Instead,
the dose required was 2 5 g per kg   It may be that the chlorine of the calcium
salt was not available, but the plasma CO_2-combining power was as low after
administration of 1 5 g of calcium chloride per kg as it was after 2 5 g per kg
This would suggest that the ammonium salt is more toxic than the calcium, and
this must be by virtue of the ammonium ion

It seems unlikely that the renal tubular necrosis was directly related to the
acidosis, since the changes were minimal in Group 1 and irregular in their extent
in Group 2, although all animals were profoundly acidotic   The appearance of
the kidneys at post-mortem suggests that the ultimate cause of the tubular
necrosis was anoxaemia due to a deviation of the blood from the juxta-medullary
region   The cause of this deviation is not apparent, but since the avascular
zone was present in both groups, it suggests that the altered blood flow was
directly related to the acidosis

Several minor points arise for discussion   It seems from the histological
appearances and biochemical findings that fat is mobilized from the depots long
before the glycogen stores are exhausted   Fat was present in the Kupfer cells
of the liver and in the myocardium, although both organs contained small

amounts of glycogen   Although the fatty change in the myocardium may be the result of degeneration, recent work (Dible, 1934 , Dible and Gerrard, 1938) suggests that the process is always one of infiltration

It will have been noted that the serum phosphorus and calcium were both raised prior to administration of calcium chloride   In addition the CO_2-combining power of the plasma was reduced   This latter feature was also found in our ammonium chloride series, and it appears to be related to the diet of oats and bran without greens   The former changes are probably related to this mild acidosis   The change in calcium concentration seems to be secondary to that of serum phosphorus   This is suggested by the fact that urinary phosphorus increased during this period   A further significant feature is that serum phosphorus showed a further increase during the period of calcium chloride administration   These changes would appear to indicate that phosphorus plays an important role in combating the acidosis, and that probably phosphorus is mobilized from body stores

Finally, attention must again be drawn to the occurrence of pulmonary fat emboli in these experiments   The meaning of this is not apparent, but it is important to realize that the reaction of the lungs to emboli is collapse—a fact which may be of some significance in cases of post-operative collapse   It is also interesting to note the intense eosinophil reaction to these emboli

SUMMARY

The oral administration of 2 5 g calcium chloride per kg daily to rabbits causes death after four days

This is accompanied by extensive focal necrosis in the liver, and necrosis of tubules in the juxta-medullary region of the kidney

The results suggest that death may be due to ammonia poisoning, and that the increase in blood ammonia is related to the hepatic lesions

Following administration of calcium chloride glycogen stores are rapidly depleted, but before this is complete fat is mobilized from the depots

This mobilization results in histologically demonstrable deposits of fat in the Kupfer cells of the liver and in the myocardium, and it is accompanied by the formation of fat emboli in the lungs

The fat emboli are accompanied by a marked eosinophil reaction and cause collapse of the pulmonary alveoli

There is a retention of phosphorus after calcium chloride administration and the calcium-phosphorus ratio is diminished

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ANTIBIOTICS FROM ASPERGILLI

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A SUMMARY of the work of a number of investigators on the antibiotics produced by Aspergilli has recently been made (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949) During the compilation of the tables for this summary it became apparent that some gaps remained to be filled The object of the present work was to investigate named and identified species which had not, so far, been examined for their capacity to produce antibiotics The work completes the examination, under certain conditions of growth, of strains of all known species of Aspergilli with the exception of a few so far unobtainable

Though the examination of fungi for the production of antibiotics is essentially straightforward, it should be emphasized that the statement that a given fungus does or does not produce an antibiotic must be received with a certain reserve, as conditions of growth and testing may greatly affect the result

Clearly, in a preliminary examination of a number of different cultures it is not practicable to carry out exhaustive experiments on each species In the following work a certain set of conditions was selected, and each culture tested under approximately the same conditions of growth

EXPERIMENTAL

The fungi examined were each grown on all the nine media shown in Table I, which were chosen because they have been found by many observers to allow good growth of many fungi

TABLE I — *Media Used*

1	Potato dextrose	200 g chopped potato, steamed for one hour with 600 ml water Supernatant liquid drawn off and 10 g glucose added Made up to 1000 ml with water
2	Modified Sabouraud	10 g peptone 40 g maltose 26 g malt extract Made up to 1000 ml with water
3	Modified glucose Sabouraud	10 g peptone 40 g glucose 26 g malt extract Made up to 1000 ml with water
4	Malt extract	50 g malt extract Made up to 1000 ml with water
5	Czapek-Dox	3 g $NaNO_3$ 1 g KH_2PO_4 0 5 g KCl 0 5 g $MgSO_4$ $7H_2O$ 01 g $FeSO_4$ $7H_2O$ 40 g glucose Made up to 1000 ml with water
6	Czapek-Dox + 5% corn steep	Same as Medium 5, with the addition of 50 ml corn steep liquor, neutralized and filtered, before making up to 1000 ml
7	Czapek-Dox + 5% corn steep, neutralized	Same as Medium 6, but brought to pH 7
8	Beer wort	50 ml wort, made up to 1000 ml with water
		(This medium was little used as the beer wort was difficult to obtain)
9	Yeast medium	20 g dried autolysed yeast stirred into 100 ml warm water Centrifuged and supernatant liquid poured off 50 ml of the supernatant liquid and 40 g glucose made up to 1000 ml with water

Distilled water was used to make up all the above media

The first experiments were done by growing the fungi at 24° C in 10 ml of sterile medium contained in hard glass bottles which were roughly 3 cm in diameter and 5½ cm tall Testing could only be carried out for 12 days as at the end of that time no medium remained For investigations involving more prolonged sampling the cultures were planted in 250 ml Erlenmeyer flasks containing 70 ml of sterile medium and were tested for 35 days Some slow growing members of the *Aspergillus glaucus* group were grown in hard glass bottles with extra sugar in the media 20 per cent glucose in Czapek-Dox potato dextrose or yeast medium Such fungi were allowed to grow at room temperature and were tested for up to 50 days

The metabolism fluid at various stages of growth was tested by the cylinder-plate method (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941, Heatley, 1944) with arbitrarily selected bacteria, strains of *Bact coli*, *Staph aureus*, *C xerosis* and *Myco phlei* were used Clearly, production of very specific antibiotics, such as those produced by *Bact coli* (Heatley and Florey, 1946), may have remained unobserved if an organism sensitive to them was not included

RESULTS

The results are set out in Table II

TABLE II —*Results*

Aspergillus	Strain	Bact coli	Staph aureus	Myco phlei	C xerosis	References from Table I of media on which activity was produced
A GLAUCUS group						
A repens series						
A pseudo glaucus Bloch	Baarn	—	—		—	
A ruber series						
A proliferans G Smith	NCTC 6546	—	—	—	—	
A chevalieri series						
A chevalieri (Mang) Thom and Church	NRRL 78	—	—	—	—	
A chevalieri (Mang) Thom and Church, var intermedius Thom and Raper	NRRL 82	—	+	+	+	2, 3, 4
A amstelodami series						
A itaconicus Kinoshita	Baarn	—	—	—	—	
A herbariorum series						
A carnoyi (Biourge) Thom and Raper	NRRL 126	—	—	—	—	
A mangini n comb	NRRL 117	—	+	+	+	9 with 20% glucose after 50 days
A echinulatus (Delacr) Thom and Church	Baarn	—	—	—	—	
A micro glaucus Thom and Raper (syn A glaucus)	NRRL 127	—	+	—	+	5 with 20% glucose after 35 days
A umbrosus Bainier and Sartory	Baarn	—	—	—	—	
A restrictus series						
A gracilis Bainier	Baarn	—	+	+	+	5 with 20% glucose after 50 days
A NIDULANS group						
A caespitosus Thom and Raper	NCTC 6972	—	+	+	+	5, 6, 7
A nidulans (Eidam) Wint var latus Thom and Raper	NRRL 200	—	—	—	—	
A quadrilineatus Thom and Raper	NRRL 201	—	+	+	+	5, 6, 7.
A rugulosus Thom and Raper	NRRL 206	—	—	—	—	
A unguis (Emile Weil and Gaudin) Thom and Raper	Baarn NRRL 216	—	+	+	+	5 or 1
A laricolor (Berk and Br) Thom and Raper	Baarn	—	—	—	—	

TABLE II (cont) —*Results*

Aspergillus	Strain	Bact coli	Staph aureus	Myco phlei	C xerosis	References from Table I of media on which activity was produced
A USTUS group						
A *granulosus* Raper and Thom	NCTC 6973	—	—	—	—	
A VERSICOLOR group						
A *janus* Raper and Thom	NCTC 6970	—	+	+	+	1, 5, 6 7 8
A *janus* var *brevis* Raper and Thom	NCTC 6971	—	+	+	+	1, 5, 6, 7
A TERREUS group						
A *terreus* series						
A *terreus* Thom var *aureus* n var	NRRL 1923	—	+	+	+	1 or 4
A *terreus* Thom var *boedijni* n var	NRRL 680	+	+	+	+	1, 2, 3, 4
A *terreus* Thom var *floccosus* Shih	Baarn	—	+	+	—	4
A *carneus* series						
A *carneus* (v Tiegh) Bloch	NRRL 527	—	+	+	+	1, 2, 6
A NIGER group						
A *niger* series						
A *awamori* Nakazawa	NCTC 2044	—	—	—	—	
A *foetidus* n sp	Baarn	—	—	—	—	
A *phoenicis* (Cda) Thom	Baarn	—	(+)	(+)	(+)	
A *carbonarius* series						
A *atropurpureus* Zimmerman	Baarn	—	—	—	—	
A *fonsecaeus* n sp	NRRL 67	—	(+)	(+)	(+)	
A *luchuensis* series						
A *japonicus* Saito	NCTC 5604b	—	—	—	—	
A WENTII group						
A *alliaceus* Thom and Church	Baarn	+	+	+	+	4
A *avenaceus* G Smith	NCTC 6545	—	—	—	—	
A *panamensis* Raper and Thom	NCTC 6974	—	+	+	+	1 or 8
A TAMARII group						
A *terricola* series						
A *lutescens* (Bain) Thom and Church	NRRL 425	+	+	+	+	all
A FLAVUS ORYZAE group						
A *micro virido citrinus* Cost and Lucet	NRRL 48	—	—	—	—	
A OCHRACEUS group						
A *sulphureus* series						
A *quercinus* (Bain) Thom and Church	NCTC 6979	±	+	+	+	3, 6
A *sulphureus* (Fres) Thom and Church	Baarn	±	+	+	+	3 2, 4 6
A *ochraceus* series						
A *butyraceae* Bainier	Baarn	—	—	—	—	
A *elegans* Gasperini	Baarn	—	—	—	—	
A *melleus* Yukawa	Baarn	±	+	—	+	2 3 4 6
A *sclerotiorum* Huber	Baarn	—	—	—	—	
A *sparsus* Raper and Thom	NCTC 6975	—	—	—	—	

Source of culture , NCTC = National Collection of Type Cultures England
Baarn = Centraalbureau voor Schimmelcultures Baarn Holland   NRRL =
Northern Regional Research Laboratory Peoria, U S A

Activity , + active, — inactive, ± very slightly active (—) activity probable
due to low pH as on neutralisation no activity was demonstrated

The following species of Aspergilli have not been examined, as cultures could not be obtained (1) *Aspergillus miyakoensis* Nakazawa, (2) *Aspergillus montevidensis* Talice and Mackinnon, (3) *Aspergillus humicola* Chaudhuri and Sachai, (4) *Aspergillus delacroixii* (Sacc) Thom and Church Thom and Raper (1945) considered that " it is possible that some old material of a strain of *A oryzae* might have furnished the type "

SUMMARY

Thirty-seven species of Aspergilli, comprising 42 strains which are not known to have been investigated before, have been examined for their ability to produce antibiotics in surface culture on 9 different liquid media Twenty strains produced metabolism solutions with some antibacterial activity

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THE NATURE OF SOME ANTIBIOTICS FROM ASPERGILLI

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DURING a survey of 42 cultures of Aspergilli (Gill-Carey, 1949) several species were discovered which produced antibiotics, and some of these have been investigated further

General conditions of culture

All the fungi investigated were grown in stationary cultures in glass vessels on 500 ml of medium in a layer 2 cm in depth Incubation was carried out at 24° C, the activity which developed in the medium being followed by the cylinder-plate method of assay (Heatley, 1944)

Antibiotics from the Aspergillus ochraceus group

Antibiotics were isolated from two closely related species, *A quercinus* (Bain) Thom & Church, NCTC 6979, and *A sulphureus* (Fres) Thom & Church, Baarn

A quercinus

Medium and conditions of culture—The fungus was cultivated on glucose-Sabouraud medium containing 1 per cent peptone, 2 6 per cent malt extract and 4 per cent glucose for 15 days

Isolation—The active principle was removed from the metabolism solution by shaking with 2 per cent charcoal and eluting with 80 per cent acetone

The acetone was distilled off under reduced pressure and the remaining aqueous solution shaken with ether at pH 2 On evaporation of the ether, colourless rhombic crystals were obtained and were purified by recrystallization from hot water or petrol ether

Chemical properties—The substance recrystallized from petrol ether in the form of colourless needles, m p 83° C On admixture with an authentic sample of penicillic acid, m p 83° C , there was no depression in melting point The substance crystallized from hot water in the form of rhombic plates, m p 60° C

[Birkinshaw, Oxford and Raistrick (1936) report m p of 83–84° C and 64–65° C for penicillic acid recrystallized from petrol ether and hot water respectively]

The material was stable at 100° C in aqueous solution at pH 2 but its antibacterial activity slightly decreased on boiling for 30 minutes at pH 9

The substance gave no coloration with ferric chloride

With strong ammonia the substance gave the characteristic pink colour reaction for penicillic acid (Birkinshaw *et al*, 1936)

The antibacterial properties of the material agreed with the figures given for penicillic acid (Alsberg and Black, 1913 , Heatley and Philpot, 1947 , Kavanagh, 1947 , Oxford, Raistrick, and Smith, 1942)

It was thus evident that this strain of *A quercinus* produced penicillic acid

A sulphureus

Medium and conditions of culture —This species was cultivated under similar conditions to *A quercinus* The metabolism solution was harvested after 12 days' incubation

Isolation —The active principle was extracted in a manner similar to that used for the substance from *A quercinus*

Chemical properties —The substance recrystallized from petrol ether in the form of colourless needles, m p 83° C When mixed with the substance from *A quercinus* the melting point was 83° C

With strong ammonia the substance gave the characteristic pink colour reaction for penicillic acid

It was thus evident that these strains of *A sulphureus* and *A quercinus* produced the same substance, penicillic acid

Antibiotics from the A wentii *group*

Antibiotics were isolated from two species in the *A wentii* group, *A wentii* Wehmer, IMI 17295, and *A alliaceus* Thom & Church, Baarn

A wentii

Several strains of *A wentii* have been tested for antibacterial activity, one by Wilkins and Harris (1942), five by Wilkins (1944), and four by Furtado (1944) Strain 17295 of the Imperial Mycological Institute was tested and found to produce good antibacterial activity when grown on a 5 per cent malt extract broth Two other strains tested simultaneously, IMI 23010 and IMI 23012, yielded culture fluids with much weaker activity

Thom and Raper (1945) reported that a strain of *Aspergillus wentii* was found by Karow (1942) to produce citric acid Yabuta (1912, quoted from Thom and Raper 1945) reported the production of kojic acid by *A wentii*, but Birkinshaw, Charles Lilly and Raistrick (1931) stated that the culture fluid of *A wentii* gave negative tests for kojic acid Possibly these divergent results were due to the use of different strains of the organism by different workers

Medium and conditions of culture —The fungus was grown on 5 per cent malt extract broth The metabolism solution was harvested after 15 days' incubation

Isolation —The active principle was removed from the culture fluid at pH 6 by continuous extraction with ether On evaporation of the ether, needle-shaped crystals were obtained These were recrystallized from aqueous acetone

Chemical properties —The substance melted at 150° C On mixing with an authentic sample of kojic acid (Kemball Bishop & Co Ltd) m p 150° C , the melting point was not depressed

The substance gave a deep red-purple colour with ferric chloride which did not disappear on standing

The chemical and antibacterial properties of the substance agreed with those given for kojic acid (Birkinshaw, Charles, Lilly and Raistrick, 1931 ; Heatley and Philpot, 1947 ; Jennings and Williams, 1945)

It was thus evident that *A wentii* IMI 17295 produced kojic acid

A alliaceus

Medium and conditions of culture —This species was cultivated under similar conditions to *A wentii* The metabolism solution was harvested after 10 day's incubation

Isolation —The active principle was extracted in a manner similar to that used for the substance from *A wentii*, IMI 17295 It formed needle-shaped crystals

Chemical properties —The substance melted at 150° C On mixing with an authentic sample of kojic acid (m p 150° C) the melting point was not depressed

The substance gave a deep red-purple colour with ferric chloride which did not disappear on standing

The chemical and antibacterial properties of the substance agreed with those given for kojic acid It was thus evident that this strain of *A alliaceus* produced kojic acid

Antibiotics from A nidulans *group*

Penicillin production was demonstrated in two species in the *A nidulans* group, *A caespitosus* Thom and Raper, NCTC 6972, and *A quadrilineatus* Thom and Raper, NRRL 200 A second antibiotic was produced by *A caespitosus*

A caespitosus

Medium and conditions of culture —The fungus was grown on modified Czapek-Dox medium containing 0 3 per cent $NaNO_3$, 0 1 per cent KH_2PO_4, 0 05 per cent KCl, 0 05 per cent $MgSO_4$ $7H_2O$, 0 001 per cent $FeSO_4$ $7H_2O$ and 4 per cent glucose, made up with tap water

After 10 days the metabolism solution produced a zone of inhibition of about 20 mm diameter on plates seeded with the H strain of *Staph aureus* This activity was destroyed completely by the addition of a few drops of penicillinase to the test solution After 20 days, zones of about 15 mm were obtained, and this activity was not destroyed by the addition of penicillinase Zones of inhibition were just detectable after incubation for 25 days

A slight purple colour developed in the medium about the ninth day, and after 15 days it was deep purple

Some culture fluid was harvested after 10 days and some after 20 days' incubation

The antibiotic present after 10 days' incubation —The active principle was unstable Complete destruction occurred on heating to 100° C for 15 minutes at pH 2 or 9 Some destruction occurred after 30 minutes at 20° C

The active principle was extracted into ether at pH 2 (at 5° C) and back into phosphate buffer at pH 7

The activity was destroyed completely on incubation with penicillinase solution or copper sulphate solution (Abraham and Chain, 1942)

The antibiotic present after 20 *days' incubation* —The active principle was relatively stable   Destruction occurred on heating to 100° C for 15 minutes at pH 2 but not at pH 9

It was precipitated with HCl at pH 2 in association with a purple pigment

Thus a penicillin-like substance was produced by this strain of *A caespitosus* after 10 days, and was followed by another antibiotic after 20 days' incubation

A quadrilineatus

Medium and conditions of culture —This species was cultivated under similar conditions to *A caespitosus*   The metabolism solution was harvested after 15 days' incubation

Nature of antibiotic produced —The active principle was unstable   Complete destruction occurred on heating to 100° C for 15 minutes at pH 2 or *3*   Some destruction occurred after 30 minutes at 20° C

The active principle was extracted into ether at pH 2 (at 5° C) and back into phosphate buffer at pH 7

The activity was destroyed completely on incubation with penicillinase solution or copper sulphate solution (Abraham and Chain, 1942)

Thus a penicillin-like substance is produced by this strain of *A quadrilineatus*

SUMMARY

The antibiotics produced by one strain each of six species of Aspergilli were investigated   Two species from the *A ochraceus* group, *A quercinus* (Bain) Thom and Church, and *A sulphureus* (Fres) Thom and Church, were found to produce penicillic acid   two species from the *A wentii* group, *A wentii* Wehmer (strain IMI 17295), and *A alliaceus* Thom and Church, were found to produce kojic acid and two species from the *A nidulans* group, *A caespitosus* Thom and Raper and *A quadrilineatus* Thom and Raper, were found to produce penicillin-like substances   *A caespitosus* produced also another antibiotic

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THE EFFECT OF TRIPHENYLMETHANE DYES ON THE INTRA-CELLULAR GROWTH OF INFLUENZA VIRUS A [*]

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IN a recent study of the growth cycle of influenza virus A in the fertile egg (Hoyle, 1948) it was shown that growth of virus occurred in a series of intra-cellular cycles, each cycle lasting about 6 hours   Infective red cell agglutinating virus introduced into the allantoic sac was rapidly taken up by the cells of the chorio-allantoic membrane, but appeared to be converted into a non-infective and non red cell agglutinating form   This intracellular form of the virus then multiplied for a period of 6 hours, at the end of which time a much larger amount of infective virus appeared in the membrane and was almost immediately excreted into the allantoic fluid   Although virus could not be demonstrated in the chorio-allantoic membrane by agglutination tests during the period of intracellular multiplication, nevertheless the virus multiplication could be followed by reason of the appearance and rapid increase in amount of complement-fixing soluble antigen in the membrane during this period

It was suggested that the influenza virus existed in two forms, an extracellular infective form which agglutinated red cells, and an intracellular multiplying form which did not agglutinate red cells and was possibly identical with the soluble antigen   Whether this theory was correct or not, it seemed evident that the production of soluble antigen in the chorio-allantoic membrane was related to the intracellular multiplication of virus and could be used as a measure of that multiplication   If eggs were inoculated to the allantoic sac with a large dose of

virus sufficient to infect simultaneously all the available cells, then the production of soluble antigen in the chorio-allantoic membrane was remarkably constant, both in rate of production and total amount produced   It seemed that there was here available a very convenient technique by means of which the effect of any substance on the intracellular growth of the influenza virus could be studied   This paper describes the results of such experiments with a variety of dyes, in particular with dyes of the triphenylmethane group

Technical Methods

A highly egg-adapted strain of virus (D S P) was used throughout the work   Twelve-day-old fertile eggs were inoculated to the allantoic sac with 0 3 ml of undiluted allantoic fluid from an egg inoculated 18 hours previously with D S P virus   This very large inoculum was more than sufficient to infect all the available cells   The eggs were incubated 30 minutes to allow infection to take place, and then the dye to be tested was inoculated to the allantoic fluid   The eggs were then incubated for a further $5\frac{1}{2}$ hours, and the chorio-allantoic membranes removed, washed in saline to remove allantoic fluid, suspended in 1 ml of saline containing 0 08 per cent sodium azide, and frozen and thawed three times to liberate the soluble antigen   The fluids were then centrifuged and the supernatants tested for soluble antigen content by complement fixation with human convalescent serum   It was found to be unnecessary to remove infective virus from the fluids by red cell adsorption before carrying out the complement-fixation tests, since in these experiments with very heavy inocula the amount of infective virus produced in 6 hours was too small to give appreciable complement-fixation, practically the whole of the complement-fixing power of the extracts being due to soluble antigen

In each experiment control eggs were tested in which no dye was added   In 30 such control eggs the average titre of complement-fixing antigen in the membrane extract at 6 hours was 1 36 with extremes of 1 14 and 1 80   It was therefore considered that in the experiments with added dyes a titre of 1 10 or less could be taken as indicating a significant reduction of intracellular growth   The titre was taken as that concentration of antigen giving 50 per cent fixation and was determined by the methods described by Hoyle (1945)

Preliminary Experiments

Attention was first directed to the possible effect on intracellular growth of virus of dyes commonly used in vital staining methods   A range of such dyes was tested in doses of 1 mg   The dosage corresponded to a concentration of dye in the allantoic fluid of about 1 10 000   It was found that at this concentration all the acid dyes tested were non-toxic to the embryo and none produced any effect on the production of soluble antigen   Basic dyes proved to be more toxic, and in some cases the test had to be repeated with smaller doses of dye   With dahlia violet it was found that the production of soluble antigen was markedly reduced even by doses of only one-tenth of the toxic dose   This observation led to a special study being made of the basic dyes of the triphenylmethane group

TABLE I.—*Titration of Complement-fixing Antigen in Chorio-allantoic Membrane Extracts of Eggs Infected 6 Hours Previously with a Large Dose of Influenza Virus A, the Eggs being Treated 30 Minutes after Inoculation with Varying Amounts of Dahlia Violet*

| | Amount of dahlia violet (mg) | State of embryo at 6 hours | Antigen control | Haemolysis with antigen dilutions of— | | | | | | | Antigen titre |
				1:1	1:2	1:4	1:8	1:16	1:32	1:64	
1	0	Alive	++++	-	-	-	-	-	-	++++	48
2	0.5	Dead	++++	+++	+++++	+++++	+++++	+++++	+++++	++++	nil
3	0.25	Alive	++++	++	+++++	+++++	+++++	+++++	+++++	++++	1
4	0.125	,,	++++	-	-	-	+++++	+++++	+++++	++++	6
5	0.06	,,	++++	-	-	++	+++++	+++++	+++++	++++	4
6	0.03	,,	++++	-	-	-	++++	+++++	+++++	++++	6
7	0.015	,,	++++	-	-	-	-	+	+++++	++++	20
8	0.0075	,,	+++++	-	-	-	-	-	+++	++++	28

Serum: Human convalescent titre 1:16, used 1:3, control ++++

++++ = Complete haemolysis, +++, ++, + = intermediate degrees of haemolysis, - = no haemolysis.

The following is a list of dyes which were found to be without effect on soluble antigen production in doses of 1 mg

Acid fuchsin   Neutral red
Brilliant cresyl blue   Phenolphthalein
Chlorazol black   Phenolsulphonephthalein
Isamine blue   Rosolic acid
Janus green   Trypan blue
Light green   Vital new red
Methylene blue

Reduction of Soluble Antigen Production in the Chorio-Allantoic Membrane by Basic Dyes of the Triphenylmethane Group

Table I shows the results of titrating the complement-fixing antigen in chorio-allantoic membrane extracts from infected eggs treated with varying amounts of dahlia violet 30 minutes after inoculation   The dye proved to be toxic to the embryo in doses of 0 5 mg , but considerable suppression of soluble antigen production occurred even in doses of 0 06 or 0 03 mg
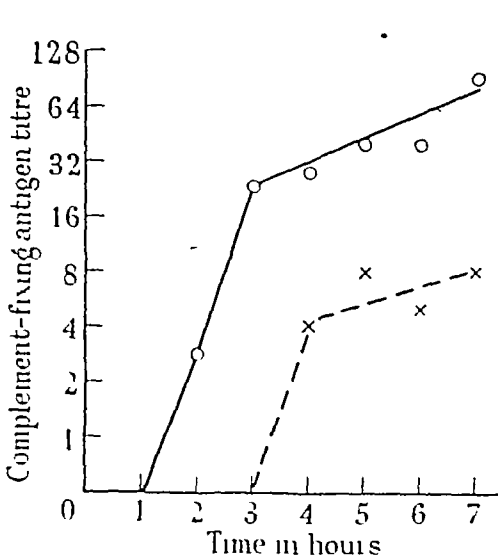

FIG 1

FIG 2

FIG 1   Effect of 0 1 mg of dahlia violet added 30 min after inoculation on rate of production of soluble antigen in chorio allantoic membranes of eggs inoculated with a large dose of influenza virus A
                    Control eggs without addition of dye o————o
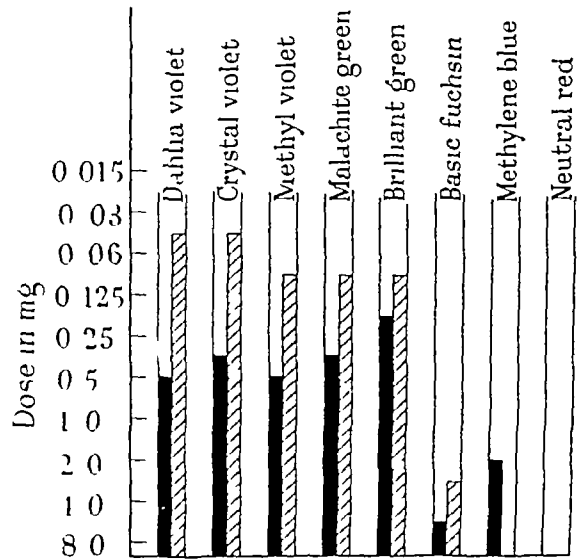   Eggs treated with dahlia violet x - -x

FIG 2 —Effect of basic dyes on production of soluble antigen in chorio allantoic membrane   Doses toxic to embryo in 6 hours indicated in solid black   Doses producing significant reduction of soluble antigen production indicated by shading

Fig 1 shows the effect of 0 1 mg of dahlia violet on soluble antigen production at various intervals of time   A batch of eggs was inoculated with 0 3 ml D S P virus allantoic fluid and 30 minutes later 0 1 mg of dahlia violet was added to half the eggs   Eggs were then sampled at hourly intervals, and the soluble antigen

content of the chorio-allantoic membranes measured The production of soluble antigen was reduced and retarded in the eggs treated with dahlia violet

Similar experiments were done with other basic triphenylmethane dyes and the results are shown in Fig 2, with, for comparison, results obtained with methylene blue and neutral red All six of the basic triphenylmethane dyes produced significant reduction in soluble antigen production in doses which were non-toxic to the embryo, but the dyes were not equally effective The violet dyes gave the best results, the green dyes were more toxic and less effective, while basic fuchsin was much less toxic than the other dyes and was only very slightly effective Methylene blue had no effect on soluble antigen production even in doses which killed the embryo in 6 hours With this dye the embryo usually survived for some hours even after the addition of toxic doses, and in this time production of soluble antigen occurred Neutral red was non-toxic even in doses of 8 0 mg and had no effect on soluble antigen production

Nature of the Effect of Basic Triphenylmethane Dyes on Soluble Antigen Production

The experiments described show that in suitable doses certain basic dyes of the triphenylmethane group will greatly reduce the production of soluble antigen in the chorio-allantoic membrane of eggs inoculated with influenza virus A This effect might be produced in three ways

(1) The dye might prevent infection of the cells

(2) The dye might destroy the soluble antigen

(3) The dye might interfere with the metabolism of the infected cell in such a way as to reduce the intracellular multiplication of the virus

In all the experiments the eggs were inoculated with a very large dose of virus, and a period of 30 minutes allowed before addition of the dye Under these circumstances it seems impossible that the dye should have been able to prevent infection of the cells Also the red cell agglutinating power of virus is unaffected by concentrations of dye which are effective in suppressing soluble antigen production in the infected egg Thus a concentration of crystal violet of 1 100,000 in the allantoic fluid markedly reduced soluble antigen production, but it required at least ten times this amount to produce any effect on the ability of virus to agglutinate red cells The infectivity of the virus was also unaffected by 30 minutes' exposure to 1 100,000 crystal violet, a 1 20 dilution of D S P virus allantoic fluid so treated was still infective in a dilution of 1 100 million The infectivity was considerably reduced by 1 10,000 crystal violet and was destroyed by 1 1000 The soluble antigen is even more resistant than the agglutinin *in vitro*, thus with crystal violet it required a concentration of 1 2000 to reduce the complement-fixing power of a chorio-allantoic membrane preparation of soluble antigen by 50 per cent It seems probable, therefore, that the dyes do not act directly either on the infective virus or on the soluble antigen, but that the effect is due to an interference with the metabolism of the infected cell It is probable that these dyes do have a pronounced effect on cell metabolism, since they are more toxic to the embryo than the other dyes tested

Effect of Basic Triphenylmethane Dyes on Production of Infective Virus in Eggs

Since the basic triphenylmethane dyes appear to retard and reduce the intra-cellular multiplication of virus, it might be expected that the liberation of extracellular infective virus into the allantoic fluid would also be reduced   Eggs were inoculated with 0 1 ml of a 1 10 dilution of D S P virus allantoic fluid, and 30 minutes later 0 1 mg of dye was added   The allantoic fluid was sampled at intervals and the red cell agglutinin titre measured by the Salk test, and compared with control eggs without dye   The results are shown in Fig 3   The liberation of virus into the allantoic fluid was retarded in the eggs treated with dye, though the final titre attained was not greatly reduced   It was noted in these experiments that the dyes were fairly rapidly destroyed in the egg   Thus, with crystal violet,
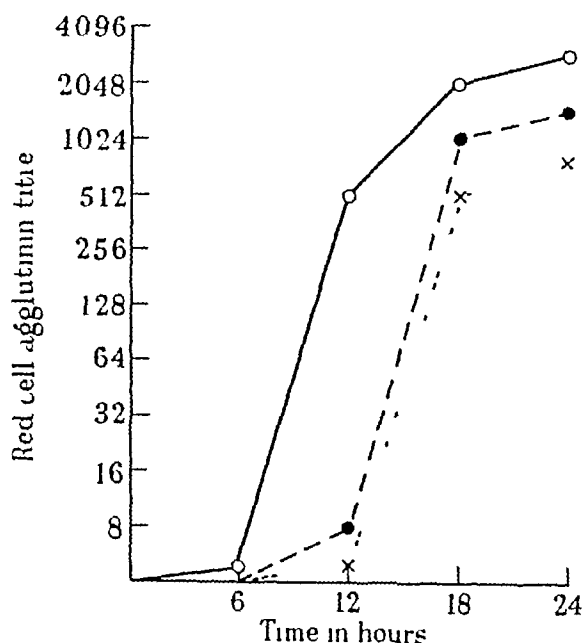

Fig 3 —Effect of 0 1 mg of crystal violet and 0 1 mg of malachite green added 30 minutes after inoculation on the rate of liberation of virus into the allantoic fluid in eggs inoculated with a small dose of influenza virus A

Control eggs without dye o————o
Eggs treated with malachite green ● - - - - ●
Eggs treated with crystal violet x   x

the chorio-allantoic membrane was deeply stained at 6 hours, but by 24 hours all staining had disappeared   It is probable that the intracellular multiplication of virus is delayed until the concentration of dye is reduced below the effective level, and that after this, multiplication proceeds normally so that the final titre of virus in the allantoic fluid is only a little less than in the controls

DISCUSSION

The experiments described in this paper show that basic dyes of the triphenyl-methane group, introduced in suitable doses into the allantoic sac of eggs infected previously with influenza virus A, are able to retard and reduce the intracellular

growth of the virus, as shown by a reduction in the production of soluble antigen in the chorio-allantoic membrane, and a retardation in the liberation of infective virus into the allantoic fluid   The effect appears to be restricted to the basic dyes of the triphenylmethane group, the violet dyes being most effective   These dyes, however, are more toxic to the embryo than other dyes, and as the effect is only shown by doses of about one-eighth to one-tenth of the toxic dose, it is unlikely that the dyes would have any therapeutic value in influenza   In fact, Andrewes, King and Van den Ende (1943) have already found them to be without chemotherapeutic value in mice infected with influenza virus

The basic triphenylmethane dyes differ from other dyes in their ability to combine with ribonucleic acid   It is to this property that they owe their efficacy in Gram's method of staining bacteria (Henry and Stacey, 1943)   Knight (1947) has shown that influenza virus A contains ribonucleic acid   If ribonucleic acid is an essential constituent of the influenza virus, then it would seem possible that the effect of basic triphenylmethane dyes in retarding the intracellular growth of influenza virus A might be due to an interference with the metabolism of ribonucleic acid in the infected cell

SUMMARY

A method is described whereby the effect of any substance on the intracellular growth of influenza virus A can be readily observed by measuring the effect of the substance on the production of complement-fixing soluble antigen in the chorio-allantoic membrane of eggs inoculated with a large dose of virus

It is shown that basic dyes of the triphenylmethane group, especially dahlia violet and crystal violet, can, in suitable doses, greatly retard and reduce the intracellular growth of the virus   These dyes are, however, more toxic than other dyes to the embryo, and it is unlikely, therefore, that they would be of any chemotherapeutic value in influenza

It is suggested that the effect of these dyes may be due to an interference with the metabolism of ribonucleic acid in the infected cell

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AN ANTIBIOTIC PRODUCED BY *STAPHYLOCOCCUS AUREUS*

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MANY reports have appeared during the last sixty years of the inhibitory or lytic power of staphylococci   In some cases it is clear that an antibacterial substance was concerned, but its properties have varied widely, being sometimes thermostable and sometimes very easily destroyed by temperatures below 100° C , sometimes soluble and sometimes insoluble in alcohol and other organic solvents , sometimes produced only on solid or on liquid medium and sometimes equally well on either   As this literature has been reviewed elsewhere (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949) it is not proposed to consider it further here

Jennings and Sharp (1947) of this laboratory examined several strains of staphylococcus for antibacterial activity, using the streak-plate test with *C xerosis*, *C diphtheriae*, *Bact coli* and *Staph aureus* NCTC No 6571 as test organisms   Twenty-six of the 205 strains so examined were found to be active and from one of them, No E 755, an attempt was made to extract the antibiotic The results of this work were summarized by Florey *et al* (1949, Chap 11) as follows

' It was found   that an abundant supply of oxygen was necessary for the production of an antibiotic in liquid culture " This had been reported independently by Magrassi and Spiga, 1946   " It was not, however, possible to produce it by bubbling either air or oxygen through liquid cultures, for it was subsequently discovered that the act of bubbling through a gas, even nitrogen inactivated a solution of the inhibitor   The difficulty was overcome by maintaining an atmosphere rich in oxygen over the surface of the culture

It was found that production was most satisfactory in Lemco broth, and was slightly improved by the addition of 1 per cent lactic acid (neutralized) Activity was judged by the inhibitory action against *C xerosis*   The active substance was not destroyed by boiling for 5 minutes at pH 8   It could be precipitated by addition of an equal volume of saturated ammonium sulphate though with some loss in activity   Treatment of the dried residue from an aqueous solution with 80 per cent alcohol, followed by evaporation of the alcohol and resolution in water, caused complete inactivation   At pH 4 5 a considerable amount of protein material was precipitated from the medium while all activity remained in solution   Adsorption on to alumina (both untreated and acid-washed) occurred, but attempts to elute with water were unsuccessful   The substance was not dialysable and could be concentrated satisfactorily from Lemco broth or gelatin digest medium by pressure filtration through cellophan

The present paper reports the results of further work on this problem

EXPERIMENTAL

Production of the Antibiotic in Liquid Medium

Media

Lemco broth and casein hydrolysate medium were both satisfactory with regard to growth of the organism and antibiotic production   The level of activity reached was slightly higher in Lemco broth, but the high protein content of this medium was a disadvantage, as far as purification of the antibiotic was concerned, in view of the probable protein nature of the latter   However, it was used in preliminary experiments to determine the properties of the antibiotic and the best method of culture of the organism

Lemco broth —This contained Lab Lemco 1 per cent, Eupepton 1 per cent and sodium chloride 0 5 per cent, made up in tap water, the pH being 7 6   In early experiments lactic acid (1 per cent, neutralized) was included, but it was later ascertained that its presence did not enhance production and it was subsequently omitted

Casein digest medium —The use of casein hydrolysate as a source of amino acids provided a medium initially free from material which was precipitable by the reagents used in concentrating the antibiotic   A tryptic digest was prepared as follows

Light, white, soluble casein (180 g) was dissolved with the aid of a mechanical stirrer in 3 l of tap water containing 10 ml of 40 per cent caustic soda solution The pH was adjusted approximately to 9 0 and 15 g of commercial trypsin were stirred in   The mixture was transferred to stoppered flasks, which were placed in a water bath at 52° C , toluene being added as a preservative   Digestion was generally allowed to proceed for 24 hours at this temperature, although a 6-hour digest (to which no toluene was added) appeared to be equally satisfactory Alkali was added at intervals during the first few hours of the digestion period to maintain the pH between 8 0 and 9 0   In the later stages there was no further tendency to shift to the acid side   Small amounts (0 5–1 0 g) of trypsin were added from time to time to replace any that became inactivated at the high temperature of the mixture

At the end of the digestion period the mixture was adjusted to pH 6 5 and after removal of the precipitate of undigested casein it was boiled until the volume had been reduced to half   This inactivated the trypsin and eliminated the toluene   The resulting mixture was filtered hot through a coarse filter paper and was made up to 6 l (≡ 3 per cent casein)

Addition of the following salts completed the medium

$$K_2HPO_4 \qquad \cdot \qquad \cdot \qquad 27\ 0\ g$$
$$KH_2PO_4 \qquad\qquad\qquad\qquad 2\ 7\ g$$
$$FeSO_4\ 7H_2O \qquad\qquad\qquad 0\ 3\ g$$

As initial alkalinity of the medium tended to result in a better yield of the antibiotic, it was adjusted to pH 7 5 before autoclaving and the copious flocculant precipitate removed by filtering through a coarse filter paper   The inclusion of glucose (0 25 per cent) in the medium before autoclaving caused an intense brown colour to develop, so this ingredient was autoclaved separately as a 25 per cent solution which was added afterwards with sterile precautions

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Method of culture

The most effective method of production, and the one which was adopted as a routine procedure, was to grow the organism in shallow layers of medium in vessels equipped to permit continuous or intermittent passage of oxygen over the surface of the medium, but not allowing bubbling through the medium itself

The organisms were grown in the rectangular porcelain vessels originally used for the production of penicillin (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941) The spout of each vessel was fitted with a rubber stopper pierced by glass inlet and outlet tubes and a third tube to which was connected a glass bulb plugged with cotton wool This was used to hold the
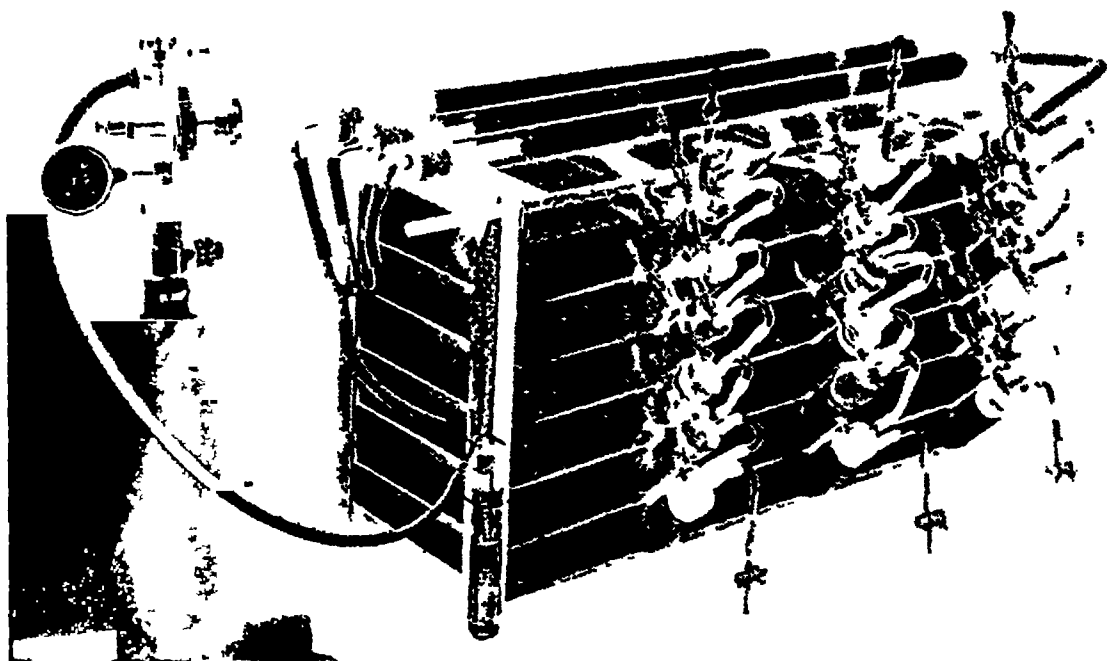

Fig 1 —Apparatus used for the culture of *Staph aureus* No E 755 in shallow layers of liquid medium under an atmosphere rich in oxygen

concentrated glucose solution which was allowed to run into the vessel after autoclaving by releasing a screw clip on the rubber connection The inoculum was introduced by the same route Each vessel contained 300 ml of medium, which formed a layer of 0 5 cm deep, this being the maximum depth which consistently resulted in good production (Fig 1)

A metal frame was designed to hold 18 vessels in 3 piles of 6 The vessels of each pile were connected in series so that oxygen entered the topmost one and passed through each in turn The bottom vessel of each pile was fitted with a glass tap by means of which the oxygen outlet tube could be closed Each pile of vessels had its own cotton-wool filter 30 inches long, the three filters being connected by means of a manifold to a single wash bottle and thence to the

oxygen cylinder   The apparatus was completely assembled before autoclaving except that the connection between the wash bottle and the manifold was left open to ensure efficient sterilization of the filters   The taps on the oxygen outlet tubes were also left open, cotton-wool plugs being inserted to prevent contamination   It proved necessary to wrap the necks of the vessels with lint and cotton-wool so as to cover the rubber stoppers, and also the outlet tubes and taps, in order to prevent occasional contamination during the culture period

After inoculation the apparatus was incubated at 37° C for 5 days, oxygen being passed through at a rapid rate for five minutes once each day   Increasing the frequency of this procedure did not enhance production

Method of assay

The cylinder-plate method (Abraham *et al*, 1941) was used, Lemco agar plates being surface-seeded with *C xerosis*   The zones of inhibition were characterized by clearly-defined edges and no resistant colonies appeared   It was not feasible to set up a standard of this antibiotic, but sufficiently accurate results were obtained by measuring the zone diameters from two-fold dilutions of the solutions to be assayed, with particular attention to the highest dilution at which any inhibition was observed   The curve relating zone diameter to dilution was rather flat

At the end of five days' incubation the culture fluid generally produced a zone of inhibition of 16 mm diameter and gave a small but measurable zone when diluted 1 in 8

Extraction and Concentration of the Antibiotic

Addition of concentrated hydrochloric acid to either a Lemco or casein hydrolysate culture until its pH had fallen to 3 0 caused a copious precipitate of inactive material to form   This was removed, together with the bacteria, by centrifuging and the clear supernatant, when neutralized, contained all the activity

The procedure for concentration of the antibiotic from cultures which had been clarified in this way varied according to the medium in which the organism had grown

Concentration from Lemco medium

Tenfold concentration was brought about with negligible loss of activity by evaporation from a shallow tray in a current of air at 56° C in a Faust-Heim apparatus   The concentrated solution, after being freed from solid material deposited during evaporation, yielded a copious precipitate when adjusted to pH 3 with concentrated hydrochloric acid   Excess acid caused it to redissolve If the solution was at least ten times as concentrated as the original culture fluid, this precipitate contained all the activity   It redissolved in water at pH 7 to give a clear, dark brown solution which gave larger and more clearly defined zones of inhibition than did the concentrated solution before acidification, possibly because of less interference with diffusion   Addition of an equal volume of 10 per cent trichloracetic acid to this solution of the antibiotic precipitated the latter with relatively little loss

Concentration from casein hydrolysate medium

When formed in this medium the antibiotic was not stable to evaporation at 56° C , and trichloracetic acid was therefore added to the neutral culture fluid, which had been clarified by centrifuging at pH 3, as described above   The details of the method were as follows

Sufficient trichloracetic acid was added (dissolved in a small volume of water) to give a final concentration of 6 per cent   The slightly cloudy mixture was allowed to stand 15 to 30 minutes and was then centrifuged   The small precipitate could be dissolved in one-sixtieth of the original volume of distilled water by bringing the pH to 7 0   It usually contained from 25 to 50 per cent of the original activity—the higher the activity of the culture fluid, the better was the relative yield on precipitation

The redissolved precipitate was dialysed against running water for at least 6 hours (dialysis could be continued for 18 hours without noticeable loss occurring), a process which halved the total solids without loss of activity   A further small amount of inactive material was eliminated by placing the dialysed solution (contained in thin-walled test tubes) in a boiling water bath for 3–5 minutes, cooling rapidly and removing the inactive precipitate by centrifuging

The solution was then dried from the frozen state, yielding about 0 1 g of voluminous, almost white, non-hygroscopic material per litre of original culture fluid

Addition of an equal volume of a saturated solution of ammonium sulphate to a cooled neutral solution of the active substance (10 mg /ml) yielded a precipitate which could be redissolved, dialysed and dried from the frozen state   It contained twice as much active substance per unit weight as did the material precipitated by trichloracetic acid, but the procedure involved disappearance of half the total activity   A more bulky precipitate resulted when the precipitation was carried out at pH 3, but the total yield was no higher

Stability and Properties of the Antibiotic

Variations in temperature and hydrogen ion concentration

The active substance was characterized by considerable stability towards heating at neutral or acid pH values, but was rapidly destroyed if heated in alkaline solution   Cultures could be rendered sterile by holding at 70° C for 20 minutes without loss of activity   Dilute and concentrated solutions derived from casein hydrolysate cultures were unaffected by heating in a boiling water bath for periods up to 1 hour at pH 3 0, or for at least 10 minutes at pH 7 0   Some activity remained even after autoclaving at 120° C for 20 minutes at pH 3 0   In general the stability seemed to be slightly greater at pH 3 than at pH 7

Between pH 7 and pH 9 no decrease of activity occurred during one hour at room temperature, but on heating in a boiling water bath the loss was rapid and complete   Above pH 9 loss of activity was noticeable at room temperature, hence, when neutralizing solutions care had to be exercised in order to avoid adding excess alkali

Action of proteolytic enzymes

The action of commercial and crystalline trypsin at 37° C , pH 8 and in concentration of 1 mg per ml resulted invariably in total inactivation within five

minutes of solutions containing up to 10 mg of dried preparations of the antibiotic per ml   The action of 1 per cent pepsin at pH 3 was not so rapid, as up to 25 per cent of the original activity remained after 30 minutes at 37° C , and traces still remained after 1 hour

Bubbling and mechanical agitation

As has already been reported by Florey *et al* (1949), bubbling nitrogen or air through an active solution under sterile conditions brought about its inactivation The destruction was a gradual process being complete in 3 hours in the case of a culture in either Lemco or casein digest medium   The same result was obtained at pH 3 and also in the presence of protective agents, such as glycine or gelatin

Mechanical shaking did not appear to exert such a marked destructive affect as did bubbling, and culture fluids proved to be unaffected by the brief though intense foaming that occurred during passage through a Sharples centrifuge at an acid pH value

Concentrated solutions of the antibiotic were less rapidly inactivated by bubbling and shaking than were the crude culture fluids, as might be expected if the inactivation were due to a surface denaturation

Drying

A cell-free Lemco broth culture withstood evaporation to dryness in a current of air at 56° C , though with casein hydrolysate cultures inactivation occurred under these conditions   Concentrated solutions of active material which had been precipitated in various ways could be dried from the frozen state without loss, but this was not the case with dilute solutions   The dried material could be stored in the refrigerator for several weeks without loss of activity taking place

Dialysis

Concentrated solutions could be dialysed in cellophan bags at room temperature for at least 15 hours without loss   Dilute solutions sometimes underwent loss under these conditions, but this was possibly due to the intervention of other agencies, such as contaminating bacteria

Filtration

Solutions could be passed through a sintered glass bacterial filter with little loss of activity, but gradocol membranes, like asbestos filter pads, removed the greater part of the active substance

Ammonium sulphate

Addition of ammonium sulphate either in solid form or as a saturated solution, and under various conditions of concentration, temperature and pH to a clarified culture fluid resulted in complete loss of activity from both the precipitate and the remaining liquid   A concentrate from Lemco broth behaved in a similar manner, though in the presence of high glycine concentrations (up to 2 M) half the activity could be demonstrated in the precipitate   The remainder had disappeared   Treatment of a concentrated solution of the dried material obtained by precipitation with trichloracetic acid from casein hydrolysate cultures, with ammonium sulphate in the absence of glycine gave a 50 per cent yield in the precipitate

Organic solvents

The antibiotic was characterized by marked instability towards organic solvents, especially in dilute solution   As the degree of purification increased greater apparent stability in the presence of certain solvents was noted

Culture fluids, or dried residues from them, could not be treated with alcohol at 4° C without total loss of activity taking place   The material precipitated by trichloracetic acid was inactivated less rapidly in the presence of alcohol in concentrations up to 70 per cent, though the extent of the loss was variable and 80 per cent alcohol caused total inactivation   The antibiotic was soluble in 70 per cent ethyl alcohol, but, owing to its destruction, it was not possible to determine whether or not it was soluble in absolute alcohol and in various other solvents Methyl alcohol, butyl alcohol and acetone exerted a similar deleterious effect

If a solution were shaken gently for a few minutes with an equal volume of ether, little activity could be detected in either the aqueous or ether fraction though, as was the case with alcohol, there was evidence of apparently increasing stability of the antibiotic, as more concentrated solutions became available

Chloroform exerted the most marked destructive effect of all the solvents tested, irrespective of the degree of concentration and purity of the solution Introduction of a few drops, without shaking, brought about loss of half the activity almost immediately and inactivation was complete within 7 hours

The known properties of the antibiotic suggest that it is an unstable protein of relatively high molecular weight   The impure state of the preparations used makes it impossible to draw any more definite conclusion

Antibacterial Activity

The selective antibacterial action, and the fact that activity could be demonstrated on a medium containing catalase, indicated that the active agent was a specific antibiotic substance and not hydrogen peroxide (Jennings and Sharp, 1947)   This was confirmed in the case of the active substance produced in liquid media   culture fluids and concentrates derived from them both exhibiting equal activity with and without the addition of blood as a source of catalase

The antibacterial action of the concentrated active substance, freed from living bacteria   was investigated qualitatively and quantitatively by the gutter-plate method (Fleming, 1929) and by dilution tests

Gutter-plate tests

In these the antibiotic preparation, in various amounts, was incorporated under sterile conditions in a strip across the diameter of an agar plate and various test organisms were streaked across the plate at right angles to it

A solution of trichloracetic acid-precipitated material which had been redissolved in one-twentieth of the volume of original culture fluid, and which gave zones of inhibition against *C xerosis* by the cylinder-plate method of 21 5 mm diameter undiluted and of 11 mm when diluted 1 in 64, was incorporated in the gutters at dilutions of 1 in 2 and 1 in 4   Inhibition of the following organisms occurred   the distance in millimetres from the edge of the gutter to the beginning of growth of the streak being shown by the figures in brackets   Each figure is the mean of 4 observations   that for the stronger solution being shown first   *M lysodeikticus* (12 12) , *Myco phlei* (8 5 6) , *C xerosis* (8, 6) , *Staph aureus,*

NCTC No 6571 (4, 2), and *B anthracis* (4, 1) Organisms tested but not inhibited at these concentrations were *C diphtheriae* (types *gravis* and *intermedius*) and *Str pyogenes*, which were tested on serum agar plates, also *Bact coli*, *Sh sonnei*, *Ps pyocyanea*, *Salm gaertneri* and *Myco smegmatis*

With higher concentrations inhibition of *C diphtheriae* could be demonstrated, solutions of dried active material containing 1, 2, 4 and 8 mg per ml and giving zones of inhibition against *C xerosis* in cylinder-plate tests of 20 mm, 21 5 mm, 23 5 mm and 25 mm diameter respectively, were mixed with one-tenth of their volume of serum and one-fourth of their volume of melted 6 per cent agar and the mixture was filled into gutters

Table I lists the inhibitions recorded

TABLE I —*Activity against Various Organisms as Indicated by Gutter-plate Tests*

Concentration in gutter Mg per ml	Distance (mm) from edge of gutter to which inhibition extended			
	Bact coli	*C xerosis*	*C diphtheriae* (*gravis*)	*Staph aureus*
0 7	0*	6 0	5 0	3 5
1 4	1 5	5 0	7 0	4 0
3 0	2 5	6 5	8 0	5 5
6 0	3 5	8 0	8 5	6 5

* Growth over the gutter itself was inhibited, however

Comparative tests were carried out with solutions of active material precipitated by trichloracetic acid from liquid cultures and from an extract of solid medium on which the organism had grown, in order to ascertain whether the latter contained any additional active substances which were not being produced in liquid medium This did not appear to be so, as both solutions gave similar zones of inhibition of plates seeded with *C xerosis* and were found by the gutter-plate method to act in an identical manner on *C xerosis* and on *C diphtheriae* (*gravis*), the former organism being the more sensitive Solutions which had been sterilized by filtration through sintered glass proved to be less active than those which had been immersed in boiling water for 2 minutes

Dilution tests in liquid media

These were carried out on a dialysed solution of the active substance which had been precipitated by ammonium sulphate from a solution of lyophilized trichloracetic acid-precipitated material After heating to precipitate coagulable material and removing the latter by centrifuging, the solution contained 13 2 mg of solids per ml (1 part in 77) It was sterilized by immersion for 3 minutes in a boiling water bath By the cylinder-plate method it gave zones of inhibition against *C xerosis* of 26 mm undiluted and 14 mm at a dilution of 1 in 64 (1 part in 5000)

Two-fold dilutions were made, commencing at 1 part in 770 The test was carried out with a series of organisms, the inoculum being one drop per tube (measured by Dreyer's pipette) of overnight broth cultures which were diluted as shown in Table II Ten per cent serum was included in the case of *C diphtheriae* and *Sh pyogenes* The results were read after 16 hours at 37° C and are set out in Table II In no case was the degree of activity of a high order

TABLE II—*Activity Against Various Organisms as Indicated by Dilution Tests in Liquid Medium*

Test organism	Inoculum, broth culture diluted 1 part in	Inhibited completely at 1 part in	Inhibited partially at 1 part in
Staph aureus NCTC 6571	1000	6000	—
C xerosis	100	2800	—
B anthracis	100	2800	—
Str pyogenes	10	1500	—
C diphtheriae (gravis)	10	—	770

SUMMARY

The production and properties of an antibiotic formed by a strain of *Staph aureus* have been described The substance was produced in shallow layers of liquid medium when an atmosphere rich in oxygen was maintained over the surface, but was not detected when air or oxygen was bubbled through the medium

The antibiotic appeared to be a protein which could be precipitated from the cell-free culture medium by trichloracetic acid , it could be freed from the latter by dialysis and dried from the frozen state It was relatively stable to heat in neutral or acid solution, but was rapidly destroyed by alkali, organic solvents, pepsin or trypsin

The substance inhibited the growth of *C xerosis*, *Staph aureus*, *C diphtheriae*, *B anthracis*, *Myco phlei* and *M lysodeikticus* In no case was the degree of activity, as measured by dilution tests in liquid media, of a high order

The properties of the antibiotic are such as to make it probable that it will have no therapeutic application

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ELECTRON-MICROSCOPICAL STUDIES ON THE MECHANISM OF LYSIS AND THE MULTIPLICATION OF BACTERIOPHAGE OF *BACT COLI*

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OBSERVATIONS of living specimens with the ultramicroscope, using visible light, have given valuable information on the mechanism of lysis due to the action of bacteriophage of *Bact coli* (Merling, 1938)   By growing the bacilli in the presence of phage in hanging-drop cultures on the warm stage of the microscope it was possible to observe in dark ground illumination the process of lysis from beginning to end   The results were briefly that within 20 minutes of contact individual bacilli lost their motility and their normal shape and size, swelled, in most cases at one end more than the other, sometimes becoming spherical, and then suddenly burst, releasing the cell contents, which presumably consisted of bacterial protoplasm and masses of phage bodies   Counts showed about 250 phage bodies per lysed bacillus   It was assumed that the multiplication of the phage took place *inside* the bacterial cell, though proof for this assumption was lacking, owing to the limits imposed by the optical microscope

Since then a great amount of work has been carried out by a number of workers on the same subject   H Ruska (1940), and later Pfankuch and Kausche (1940), described the tadpole shape of bacteriophage, and therewith established means of identifying phage bodies by their characteristic shape and size   Recently Wyckoff (1948) showed electron photographs in which he described " multiplication centres " and " foci," and made the statement that " apparently most of this growth and multiplication takes place in the protoplasmic masses liberated by lysis, rather than in bacteria which are still intact "

The experiments described here were performed to find how and where multiplication takes place, whether inside or outside the cell, and how many stages can be observed in the formation of the number of phage bodies that are seen on lysis and released by the bursting of one individual bacterial cell

The problems proposed for investigation were   (1) Is it possible to correlate the findings of observations on living cells, showing that between 200 and 250 phage bodies were released from one lysed bacillus with the pictures obtained in the electron microscope ?   (2) How and where does the multiplication of the bacteriophage of *Bact coli* take place ?

MATERIAL

The strains of *Bact coli* and bacteriophage were the same as used previously for living observations   Twenty-four-hour broth cultures of *Bact coli* were plated on agar, and broth containing phage was added in single drops to the

surface-dried plate following the usual technique   After incubation for 6; 12, 24 and 36 hours, specimens were prepared from the edge of plaques and other parts of the plaques and plate   By taking specimens at increasing distances from a single plaque in the centre of a plate culture, earlier stages of lysis could be found than in samples taken simultaneously from the plaque edge   Other specimens were prepared by making drop cultures of *Bact coli* and bacteriophage on the membraned grid of the electron microscope, and were examined at times of contact varying between 1 and 45 minutes   In these specimens the material had to be mounted in doubly distilled water, after experience had shown that broth suspensions were unsuitable for examination in the electron microscope, and further that accurate timing of contact was largely impossible owing to the lag introduced by the repeated washing necessary to remove salts from the specimen   The timing of contact and process of lysis was carried out by removing the meniscus of the drop cultures by means of micropipettes, a process which took no longer than 2 or 3 seconds

TECHNIQUE

Specimens were prepared for examination in the electron microscope in the usual way by mounting collodion membranes on copper grids   The collodion membranes were prepared by adding to the usual solution of 2 per cent pyroxilin in amyl acetate, 3 5 per cent of ethyl lactate as a plasticizer   This was suggested by Dr A Burgen as a means of obtaining membranes both thinner and of greater resistance to heat and electron bombardment than the ordinary collodion film   The membranes containing this plasticizer were highly successful not only when used immediately, but after drying in a desiccator for several days

To increase contrast the shadow-casting technique, invented by Wyckoff and Williams (1945), was used   The amount of metal evaporated and coated on to the specimen, however, was kept at a minimum to avoid distortion, the aim was to increase contrast, not the development of shadows, as it was not intended to obtain measurements in this way

Coating was carried out in a specially designed apparatus, made by Metropolitan-Vickers, which was inserted in the specimen chamber of the electron microscope   As coating materials gold, palladium and gold-palladium-alloy 40 60 were used

Hanging drop cultures were also made of all material prepared for the electron microscope and kept under observation in the living state on the warm stage of the ultra-microscope   These specimens served as controls to safeguard against misinterpretation, and to determine the exact state of the electron microscope specimen at the moment of drying

Observation in the electron microscope was greatly facilitated and improved by the introduction of a fluorescent screen (Merling, 1949) made of zinc-cadmium-sulphide by Derby and Co London, E C 2, which together with other characteristics has a $2\frac{1}{2}$–$3\frac{1}{2}$ times greater brightness than the usual Willemite screens

RESULTS

The study of bacteriophage is made difficult by the speed with which lysis of an individual bacterial cell is completed, and is further complicated by the fact

that the speed of lysis for individual cells varies from about 5 minutes to about 20 minutes after first contact of the bacterial cell with bacteriophage

Normal bacilli

To detect small changes in the appearance of the bacilli it was, of course, necessary to become thoroughly familiar with the appearance in the electron microscope of " normal " bacilli at as many stages of their development as possible

From the experience gained in this way a composite picture has been gained which shows that a " normal " bacillus during its life goes through a definite cycle   It begins as a short rod of about 0 8 or 0 9 μ in breadth, and varying in length from 1 2 to 3 μ This rod in the course of 12 hours either develops intracellular divisions which will eventually progress until separation of the daughter cells is complete, or it may deteriorate, showing granules of varying size, in which state it may be dying or dead   The very young forms, in cultures up to 12 hours old, may show very little differentiation (Fig 1), and their internal structure is restricted to shadowy areas without sharp contours   The surface layer or membrane of the bacillus can be distinguished from the internal structure by its lower scattering power for electrons, and shows as an envelope of low brightness, which in the perspective of the electron microscope appears to have a width of about 10 per cent of the breadth of the bacillus

While young forms may as a rule be regarded as mainly structureless, there are conditions, so far not determined, under which the seemingly " normal " bacilli exhibit areas of greater density, particularly at the poles, and in one or two other places   Fig 2 shows two such cells, one with polar bodies and two more spots of higher scattering power, the other probably distorted in the fortuitous perspective owing to the fast drying of the specimen

Twenty-four-hour cultures show the beginning of differentiation usually in that the greater part of the cell contents appear to contract away from the poles of the bacilli, and generally towards the central axis of the cells, thus leaving the poles more or less empty and transparent   The bacterial membrane then becomes very clearly visible and shows a structure consisting of two parts, one coarse, appearing like a branching system extending from the central axis, the other being the envelope or membrane proper (Fig 3)   Bacilli from cultures more than 24 hours old show the progressive development of more or less well defined internal structures   This differentiation, as mentioned above, may be an indication of approaching death (Fig 4 and 5)

Whilst the three appearances described successively dominate the picture according to the age of the culture, it must be emphasized that, whilst at any given moment the majority show one appearance, cells can also be found at other stages of development

It has also been possible to correlate the appearances seen in the electron microscope with those observed on living cells in the ultra-microscope   Although with the ultra-microscope it is difficult to see internal structures in the living bacilli before they have reached the state of granularity, it is just possible to glean indications that structures are present, with special methods such as the use of immersion media of a refractive index equal to or near that of the bacterial membrane (Merling, 1935)

Normal bacilli in the presence of bacteriophage

It is known from observations on living material that the first visible result of contact between a bacillus and bacteriophage is the loss of motility of the bacillus   The flagella, which normally are quite active, become motionless within a few minutes of contact and the bacillus lies still   It has, however, been very difficult to observe a bacillus in this state in specimens prepared for examination in the electron microscope

Fig. 6 shows a bacillus with some phage bodies adsorbed, but it is impossible to say whether or not one has actually entered the bacillus   The protoplasm of the bacillus is receding from the membrane unevenly

Fig 7 shows a bacillus from a 6-hour culture which has had two minutes, contact with bacteriophage   Within the two minutes seven phage bodies have been adsorbed on the surface of the bacillus and one of them has entered the cell, where it can be seen nearly surrounded by the bacterial membrane, which has presumably formed a slight extrusion, indicating entry by a foreign body , in the same picture is a small cluster of phage bodies

Fig 8 shows a bacillus which differs from the " normal " only in so far as the bacillary protoplasm has receded from the membrane evenly all round   The head of one phage body has penetrated the bacillary membrane which is beginning to form an extrusion around it

In Fig 9 the entry of one phage body into a slightly older bacillus is shown   The bacillary membrane has not yet completely engulfed the phage body, which is already dividing into two phage bodies

Fig 10 is another example of one phage body inside an extrusion of the bacterial cell   This is shown on the left of the figure underneath a small cluster of phage bodies

It has not been possible to correlate the behaviour of the flagella as seen in the living state with the picture in the electron microscope   All that can be said at the moment is that in a lysing culture there appear to be more free flagella than in the non-lysing controls, a fact which is known from observations on living cells

Bacilli infected by bacteriophage

At the moment of contact between bacillus and bacteriophage the bacillus ceases to be normal although with the present technique changes in appearance become detectable only when multiplication of phage has started within the bacillus   Two types of changes in appearances can then be distinguished   (a) those due to the ageing of the culture , (b) those due to the presence of bacteriophage and the consequent progressive lysis   These two types of appearances become superimposed

The first stage in the multiplication of the bacteriophage appears to take place at the point of entry as shown in Fig 10

Fig 11 shows the point of entry with the extrusion from the bacterial membrane enveloping a phage body in simple division   Two phage bodies are attached to the surface of the bacillus

When contact between phage and bacilli is maintained for longer than five minutes at 37° C the first and second stages of entry and multiplication at the point of entry are accompanied by further stages of development   This indicates that newly grown bacilli are continually being infected, and lysis progresses with great rapidity

Fig 12 shows a small area of greater density in the otherwise clear pole of the bacillus, which appears to be a centre of multiplication or the beginning of an intracellular colony after the entry of one phage body, although details of that multiplication cannot be discerned

In Fig 10 the bacillus, of which the greater part is shown, has two round masses at one pole, which have some structure and differ in that respect from polar bodies and other normal cell constituents

Fig 13 shows a bacillus with similar masses in which the centres are becoming slightly transparent, and Fig 14 shows several such round bodies coalescing and becoming clearer in outline It will be necessary to refer to these appearances later

In order to gain a picture of the further multiplication of bacteriophage various procedures were tried First of all it appeared promising to wash the bacilli in double distilled water in order to remove salts This was done after preparing the specimen in the usual way on the grid After one to five minutes' contact at 37° C the meniscus containing the specimen was pipetted off, and distilled water at room temperature was applied with another micropipette and sucked off, this being repeated three times The specimen was then transferred to a small desiccator, and dried quickly over phosphorus pentoxide by a water pump

This procedure did not have the desired effect of removing salts to any great extent On the other hand, it produced in some cells something that can only be regarded as an artefact, although it may be considered as similar to selective staining in light microscopy Apart from the affected cells (Fig 15-18) there were always great numbers of cells similar to those of unwashed specimens It is not known what governed this selection

The most outstanding feature presented by the affected cells (Fig 15-18) is the granularity of the bacilli On closer inspection it can easily be seen that there are two types of granules of distinctly different size, (a) granules averaging about 500 Å and (b) granules about 200 Å in diameter

Fig 15 shows a bacillus with some fairly well-defined large spherical structures inside, together with granules of the smaller type Attached to the bacillus, or accidentally positioned near it, is a spherical mass which may be interpreted either as a cell rounded up prior to bursting, or as a large extrusion from the adjacent bacillus containing a colony of phage

Fig 16 is another example of similar appearances showing granules of the larger type The arrangement of the granules is well shown and, although there seems to be mainly a random distribution, at some points semi-circular grouping can be seen

Fig 17 shows a cell with large type granules, in which part-circular arrangements of the granules can also be discerned

Fig 18 shows small type granules and contraction of the protoplasm of the cell towards one pole and one side of the cell, as well as some chromatin globules At the left pole of the same cell, in which there is no polar body, there are about 25 large type granules showing concentric semicircular arrangement

Apart from granularity another effect shown as a result of repeated washing by nearly all the figures of such specimens (Fig 16-18) is the disappearance of the bacterial envelope These effects will be considered in the discussion

The third stage of lysis consists of the disruption of the bacterial cell and the release of its contents

Fig 19 shows the outflow of the contents of a cell which has just burst at one end   Flagella, phage bodies and bacterial protoplasm can be discerned

In Fig 20 a cell is also disrupting from one end, whilst the other end, not shown in the photograph, appears to be still intact   A few phage bodies can be seen in the midst of rather coarse granules of bacillary protoplasm

Fig 21 shows the bacterial protoplasm dispersing from a cell, of which only the densest parts, i e polar bodies, etc , persist   Lysis seems to proceed by disruption of the whole cell with complete disappearance of the cell membrane This picture compares well with the appearances seen in the living specimen in the ultra-microscope   Great numbers of phage bodies can be seen in the dispersing bacterial protoplasm, which seem to consist of droplets with well-defined surfaces giving the appearance of a foam among which the phage bodies are interspersed

Fig 22 shows this bacterial protoplasm together with phage bodies adhering to it   During the disruption of this cell the contents have spread out rather more than usual, so that two negatives have had to be used to make this figure In the left upper corner can be seen a part-circular arrangement of phage bodies

The disruption of the lysed cell may take any form, varying with local conditions in the immediate vicinity of the specimen

In Fig 23 is shown part of a lysed cell in which a few phage bodies are still retained   This figure shows that the phage bodies have a tail prior to dissociation from the lysed cell   The tail cannot always be seen under such conditions owing to the very much reduced resolution brought about by the overlying protoplasm, which produces additional scattering of the electrons and therewith reduces contrast   It can easily be seen that if the resolution demanded is in the neighbourhood of 50 Å and structures separated by 50 Å are supported or overlain by a film, be it collodion or protoplasm of equal thickness within the same dimensions, such resolution can be obtained only under exceptional circumstances

Attention has already been drawn to the part-circular arrangement of phage bodies of which a few more examples are given in Fig 24 and 25, in both of which the droplet structure of the bacterial protoplasm is also apparent   Repeated observation of the part-circular grouping of phage bodies led to an attempt to discover the way in which the phages multiply

In Fig 10 13 and 14 some round masses are shown inside bacilli of more or less intact appearances   In Fig 26 a rounded-up cell is shown in which the contents appear as one mass bordered by 5 semicircles   This is interpreted as 5 colonies of phage coalescing in the centre

The inclusion body or intracellular colony need not necessarily be circular or spherical   In Fig 28 the phage bodies are of a slightly ovoid shape with no tails and are arranged altogether in two three-quarter circles, enveloped by a membrane   These are regarded as two small colonies of phage which have been discharged in this state of development from a lysing cell   A similar aspect is shown in Fig 29 and 30 in which however, the resolution is greatly reduced owing to the presence of bacterial protoplasm

The breaking up of the colonies seems to be represented in the appearance of large clusters as shown in Fig 31, and smaller clusters as shown in Fig 32 Fig 33 and 34 are further evidence of this part-circular grouping of the phages

Both figures exhibit low resolution owing to the presence of bacterial protoplasm

The tracing of the multiplication of bacteriophage so far has left out one important link, that of the development of two phage bodies from one

Fig 35 shows three groups of phage bodies, one of which is a group of five in a part-circle arrangement the two others consist of pairs of phages   Closer scrutiny of these pairs shows the central pair to consist of two complete phage bodies with tails and divisions in the heads   The division of the other pair is not clear, and only one phage has a tail   The remarkable feature of this micrograph is the close proximity of the members of the pairs, so that they seem to be flattened against each other   The resolution in this figure is very high compared to that in Fig 9 and 11, which show binary fission of phages still inside the cell The interpretation suggested is that these pairs indeed represent phages in " binary fission "   With this figure the evidence of multiplication is regarded as complete

DISCUSSION

Short survey of working conditions

In any assessment of results obtained by means of the electron microscope it is necessary to appreciate the limitations as well as the advantages of the instrument

The dehydration of the specimen in the electron microscope produces *a priori* conditions most unnatural for living organisms, and in this respect the electron microscope compares unfavourably with the light microscope   Radiation with light even when concentrated as in dark ground illumination can be rendered more or less harmless with suitable light sources and filters, whereas electron bombardment as used at present in the electron microscope cannot be varied to any great extent, apart from keeping its intensity down to the minimum necessary to make visible the structures to be observed, and to record them photographically Parallel to this bombardment goes the heating of the specimen   Both these factors in addition to dehydration may alter the specimen

When structures which are below the resolution limit of the light microscope are under observation in the electron microscope, controls in the light microscope

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EXPLANATION OF FIGURES

Bact coli and bacteriophage   Magnification about $\times$ 18,000   Fig 7, 10 15, 19, 20 shadowed with gold, all others with gold palladium alloy

Fig 11 reproduced from *Nature* by permission of the editors

PLATE I

FIG 1 —Very young " normal " (12 hour culture) bacillus showing very little internal structure

FIG 2 —Two very young " normal " (12 hour culture) bacilli showing polar bodies and other structures

FIG 3 —Two bacilli from 24-hour culture showing increasing differentiation of internal structure and structure of membrane

FIG 4 and 5 —Bacilli from older culture (36 hour) showing granules of various sizes

FIG 6 —A bacillus whose protoplasm has receded unevenly from the membrane   Some phage bodies are adsorbed, one is possibly inside the bacillus   Contact 3 minutes

FIG 7 —Bacillus (6 hour culture) after two minutes' contact with bacteriophage   Seven phage bodies have been adsorbed on to the surface of the bacillus   one phage body has entered the cell and is surrounded by the bacterial membrane   Flagella in great numbers Contact 2 minutes

FIG 8 —A bacillus forming an extrusion around one phage body   Its protoplasm has receded slightly but evenly from the membrane   Contact 5 minutes

FIG 9 —Entry of one phage body into one bacillus   The extrusion from the bacillary membrane has not completely surrounded the phage body   but this has already divided into two Contact 5 minutes

PLATE II

Fig 10 —Parts of two bacilli   Between them a small cluster of phage bodies   One phage body inside an extrusion of the bacterial cell on the top   The lower bacillus shows two round masses in the pole   Contact 10 minutes

Fig 11 —A bacillus with (on the top) two phage bodies attached to its surface and (on the lower left) an extrusion enveloping a clearly visible phage body in simple division   One thrown off phage tail lies opposite the extrusion   Contact 8 minutes

Fig 12 —A small area of greater density in the otherwise clear pole of the bacillus is a centre of multiplication after the entry of one phage body   Contact 5 minutes

Fig 13 —A bacillus with two areas whose centres are slightly transparent   These areas have some structure and differentiation which allows them to be distinguished from polar bodies and other normal cell constituents   They are regarded as intracellular colonies of phage   Contact 15 minutes

Fig 14 —A bacillus showing these areas coalescing   From plaque edge

FIG 15-18 are taken from specimens that have been washed three times with doubly distilled water

Fig 15 —A bacillus with small granules and a number of spherical areas of greater density, and attached to the bacillus a spherical mass which may be either a rounded up cell prior to bursting or a large extrusion from the adjacent bacillus containing a colony of phages   From plaque edge

Fig 16 —Two cells superimposed   High resolution at the edge of the cells shows the part circular arrangement of the granules   From plaque edge

Fig 17 —Cell with large type granules in part circular arrangements   The polar bodies seem to be disappearing   From plaque edge

Fig 18 —Cell with some small type granules in concentric semicircular arrangement   From plaque edge


PLATE III

Fig 19 —Pole of a cell in the process of bursting, discharging its contents of protoplasm and phage bodies   The beaded appearance of the flagella is due to the coarse recrystallization of the gold coating   From plaque edge

Fig 20 —A similar picture to Fig 19   In the bacillary protoplasm there are few phage bodies and large numbers of small type granules   From plaque edge

Fig 21 —Lysing cell, of which some parts are persisting whilst others disperse together with phage bodies   From plaque edge

Fig 22 —(Taken on two negatives)   Bacterial protoplasm of a lysed cell showing in the form of droplets to which numbers of phage bodies adhere   In the left upper corner the phages show part circular arrangement   From plaque edge

Fig 23 —Part of a lysed cell   The phages adhering to it have tails   Contact 30 minutes

Fig 24 —Part circular arrangement of phage bodies adhering to the protoplasm   Contact 50 minutes

Fig 25 —A similar picture of part circular arrangement of phage bodies and protoplasmic droplets   Contact 50 minutes

Fig 26 —A rounded up cell whose membrane is faintly visible   The cell contents have formed one mass with five semicircular bulges   The edges of these bulges show greater density   Contact 1 hour

PLATE IV

Fig 27 —A lysed cell showing an elongated conglomeration of phage bodies   From plaque edge

Fig 28 —Two part circular arrangements of phage bodies within a membrane   small colonies of phage discharged as a whole from a lysed cell   From plaque edge

Fig 29 —Very nearly circular arrangement of phage bodies within bacterial protoplasm   From plaque edge

Fig 30 —Similar to Fig 29   Nearly circular arrangement of phage bodies   From plaque edge

Fig 31 and 32 —Large and small clusters of phage bodies breaking away from a previously circular or semicircular arrangement   From plaque edge

Fig 33 and 34 —Part circular arrangements of phage bodies still inside bacterial protoplasm of cells just breaking up   Contact 1 hour

Fig 35 —Three groups of phage bodies one group in part-circular arrangement   the other two consisting of pairs   Resolution in this figure is very high and allows of very close scrutiny which reveals a remarkable closeness in the position of the members of these pairs, which seem to be flattened against each other   binary fission   Contact 1 hour
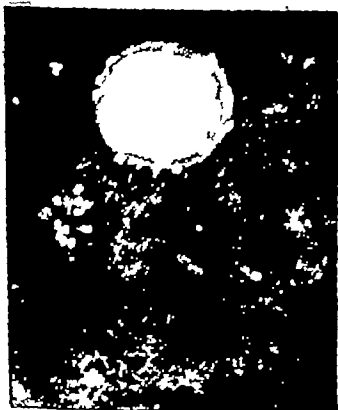
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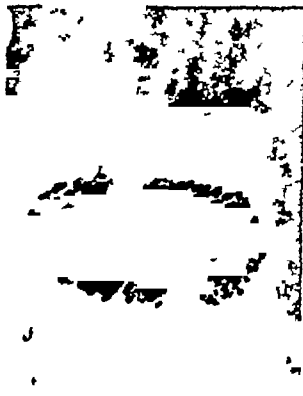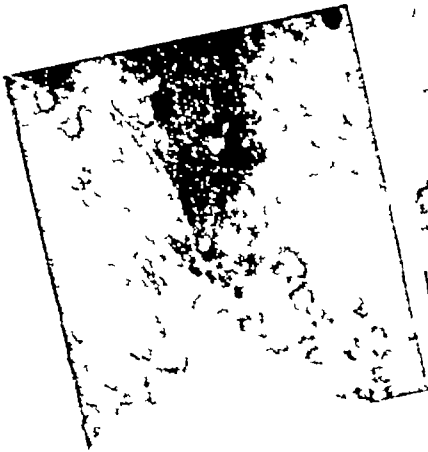Merling
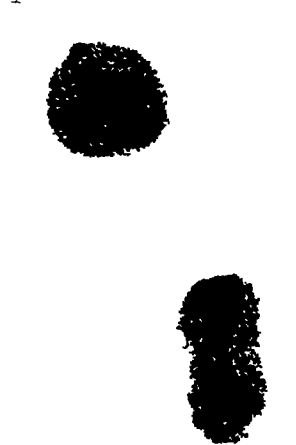
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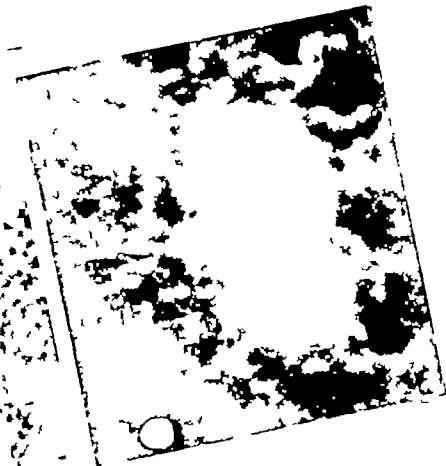11

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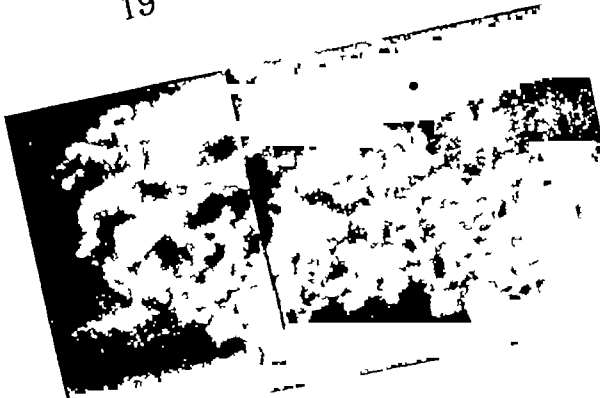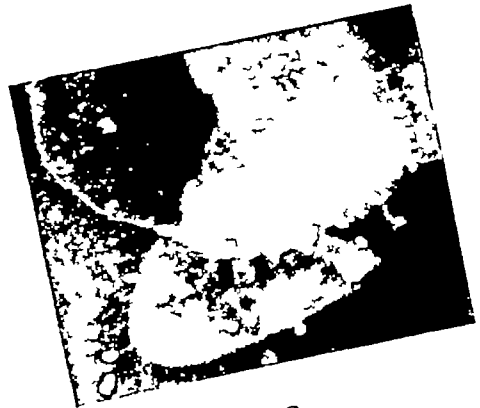
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become largely valueless   Such controls, however, have proved their worth in the study of structures that fall within, or even slightly beyond the known limits of the light microscope   It is at this point, the " limit of resolution " in the light microscope, where perhaps some progress is to be expected from knowledge gained by observations with the electron microscope

Bacteriological specimens

The loss of water which all specimens must suffer in the high vacuum of the electron microscope may have totally unpredictable results as far as biological objects are concerned   Some bacteria have been shown to be flattened out from their normal rod shape into flat ribbons , others have kept their shape, as shown by their profiles and shadows   All-round shrinking seems to occur rarely Very young forms thus may be small originally, as shown in the light microscope, or their small size may be due to a relatively greater water loss than occurs with older forms, which presumably possess a stronger envelope   Any statement with respect to the size and shape of micro-organisms and their parts must take those factors into account

Electron bombardment and heat have very distinctive effects on the specimen Observation for several minutes generally leads to loss of contrast   This is probably due to the deposit of tungsten oxide on the collodion membrane, which darkens the background   Heat may wrinkle and tear the membrane, though tears are usually due more to electron bombardment than to heat   A wrinkled and baked membrane is unsuitable for further examination   A specific effect of electron bombardment is the " building up " of fine structures into thick, coarse artefacts   The smaller the unit under observation the less will be the effect of dehydration, although heat and electron bombardment still exercise their distinct influences

In the case of *Bact coli* the drying has resulted in most cases in severe flattening of the bacterial cells, which has been apparent in profiles and shadows   On the other hand, the bacteriophage bodies do not show flattening, either in profile or by the shadowing method

The picture presented by the electron microscope of " normal " bacilli in their ordinary development from the young uniformly filled cell to the granular appearance of the old form, corresponds closely to the well-known images seen with living bacilli in the light microscope   Minor difficulties of interpretation may arise from pictures showing old forms   What is called " granularity " comprises a multitude of appearances, of which only two examples (Fig 4 and 5) are given here   In many cells two types of granules can be distinguished   (*a*) 2 or 3 large particles of about 0 3 μ in diameter, mostly situated in the poles of the bacillus, and (*b*) smaller particles of about 0 05–0 1 μ in diameter, of which there may be great numbers   In the electron microscope other cells may appear completely empty, or show only polar bodies of great density   The arrangement of the smaller particles may be similar to that shown by intracellular colonies of phages

However, it is the age of the culture that determines the proportion in which these various types of cells appear   The impression has been gained from the examination of many hundreds of specimens that the life of the ordinary bacterial cell is speeded up in the presence of phage, resulting in quick ageing and death

The first visible reaction of the majority of normal bacilli to the presence of

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bacteriophage has been stated to be their loss of motility   In the electron micro-
scope the flagella of *Bact coli* are not seen regularly, and when visible appear to
be mostly thrown off   At any rate it has not been possible to compare the
behaviour of the flagella in " normal " cells and infected cells

The first real contact between bacteria and phages appears to be a process of
adsorption   It is difficult to decide whether only one phage body can enter into
one bacterial cell   Delbruck (1945) has speculated upon this process, which he
likens to the " fecundation of the monospermic egg "   The only evidence in
favour of the one phage body one bacillus relationship is the repeatedly observed
fact that out of a number of adsorbed phages only one phage body per bacillus
has been surrounded by an extrusion of the bacillary membrane at the time of
observation (Fig 7, 9 and 11)   The formation of this extrusion around one phage
body only may be another expression of the axiom " First come first served,"
first proposed by R Salaman in the case of potato viruses X and Y, where infection
with one virus excluded the other   It has not been possible to see anything of
this process in the living specimen in the light microscope

There are similar extrusions from " normal bacilli "   Therefore the extrusion
may be   (1) an effect of drying, (2) a sign of commencing disintegration, (3) a
non-specific reaction due to local conditions, (4) a specific reaction to the entry of
a foreign body, (5) a specific reaction to the entry of one phage body

If the extrusion is specific and part of a mechanism preventing multiple
entry, a surface alteration should take place in the structure of the membrane as
a result of phage entry   On the other hand, the development of several intra-
cellular colonies in one bacillus, while not excluding their origin from one phage
entry, would suggest multiple infection

That the dense intracellular masses may be phage colonies has also been sug-
gested by Wyckoff, although he states that the greater part of growth and multi-
plication of the phage takes place in the discharged protoplasm of the dead
bacterial cell   Only improved technique can decide whether this view is possibly
correct   The present author is of the opinion that most of the phage growth
and multiplication takes place within the bacterial cell before its disruption, and
that the finding of phage bodies in clusters and adherent to discharged cyto-
plasm is due to a slowing down of the process of lysis during the quick drying
procedure, which in many cases prevents the complete separation and dispersal
of the phage bodies

That the method of preparation of the specimen has a profound influence is
shown by Fig 15–18 of specimens that have been washed 3 times with distilled
water   It appears that in the examples shown the water has had a distinct dis-
sociative action upon the cell constituents   No explanation is offered for the
frequent complete disappearance of the bacillary membrane   It is not yet known
whether this granularity represents phage colonies together with an even distri-
bution of phage bodies over the whole interior of the bacillus, but an answer to
this question may yet be found with a different technique   The sizes of granules
displayed within the cells suggest that liquefaction of some of the cell constituents
has occurred owing to the presence of the intracellular phage colonies, which can
then discharge the individual phage particles into the surrounding liquid   Fig
16 and 17 are perhaps most suggestive of this explanation   The nature of these
granules is uncertain because they are smaller than the phage bodies seen
normally   It is likely that this liquefaction is an artefact brought about by

the distilled water   The selective action of this washing on some out of a great number of cells indicates a different reaction on the part of these cells   No normal cells have shown a similar or comparable appearance after the same treatment   It is the relatively high incidence of these granulated cells that justifies the inclusion of these appearances   The moment of disruption has been caught in Fig 19 and 20   The number of phage bodies adherent to the discharged protoplasm of the lysed cell may vary considerably, there being large numbers in Fig 21, 25 and small numbers in Fig 22, 23 and 24   The greater the number of phage bodies adherent to remnants of the cell the greater is the chance of finding them in groups (Fig 21)   A frequent appearance in these specimens is that instanced in Fig 26 and 27, where the interpretation of single or multiple masses as inclusion bodies, or colonies of phage, suggests itself when the grouping of phage bodies is confirmed in definite assemblies (Fig 28, 29, 30, 33 and 34)   Coalescing colonies (Fig 26) can be recognized   The finer structure of the phage colony, persisting after the disruption of the bacillus, is shown in Fig 28, 29 and 30   Fig 28 also shows that the colony has a membrane or some sort of envelope enclosing it   The significance of colony formation by bacteriophage lies, of course, in the implication that only a living organism is capable of doing so   It is in full appreciation of this significance that the statements regarding intracellular colonies are made   It is comparatively rarely that fully transparent colonies can be found, as is only to be expected, because the resolution in a specimen must be reduced as soon as the overall thickness of the specimen exceeds a maximum value estimated at about 100 Å   Nevertheless, the grouping of the phage bodies emphasized repeatedly above can be seen easily enough   Fig 33 and 34 are further examples of this particular arrangement of phage groups, which is regarded as an indication of the mode of multiplication, of which the first step is taken to be binary fission

Wyckoff suggests that bacteriophages develop from smaller forms   If there is binary fission there ought to be no need to interpret smaller granules as smaller forms of development   Moreover, these smaller granules ought to be present in greater numbers than the fully grown phage bodies, particularly if lysis is interrupted at the right moment   This, however, appears not to be the case   On the other hand, appearances strongly suggesting binary fission can be shown both intracellularly and extracellularly (Fig 11 and 35)   Wyckoff further suggests as quoted above that " most of the bacteriophage growth takes place in the protoplasmic masses liberated by lysis," that is, after the death of the bacilli   In view of the colony formation described in this paper and experiments with other living viruses, the present author holds that the multiplication of bacteriophage takes place to its greatest amount within the bacterial cell before its disruption, although it is unlikely that the cell is still alive in the normal sense at the time of release of the virus   There is, however, a strong possibility that some phage bodies not yet fully developed at the moment of disruption of the cell will finish their development while still attached to some discharged bacterial protoplasm This process can be likened to a clockwork running down, but is not regarded as the normal mode of multiplication of bacteriophage

Smaller granules, similar to those shown in Fig 16–18, have been seen in circular or part-circular arrangement similar to the colonies shown in this communication, but they are rather isolated and uncorrelated observations and their

significance can not be assessed   These observations are therefore not included in this publication

It is suggested from the evidence produced by means of the electron microscope under control, as far as possible, of living observations in the light microscope that bacteriophage of *Bact coli* is a living organism which, like other cell-parasitic viruses, forms intracellular colonies in which the single units of the phage multiply by binary fission

SUMMARY

Experiments were carried out with *Bact coli* and its bacteriophage mainly by means of the electron microscope, but in conjunction with the ultramicroscope, in order to determine the mechanism of lysis and the multiplication of phage

It was necessary to examine first the appearances shown by "normal" bacilli in the various stages of their development   This development was found to be speeded up in the presence of bacteriophage

It was found that apparently the first sign of infection of the bacillus by a single phage body was an extrusion of the bacillary membrane engulfing one phage body whilst other phages might remain adsorbed to the surface   The second stage of the infection seemed to be the formation of intracellular colonies starting from the first entry and resulting in up to 6 such colonies to one bacillus   In the third stage, that of lysis, the bacillus burst either at one end or by disruption and disappearance of the bacillary membrane   In the discharged contents of the cell were found cell constituents like protoplasm, either as granules or as droplets, which might assume the appearance of a foam   Bacteriophage appeared to multiply by binary fission, which was seen either in freshly infected cells a few minutes after entry or in discharged clusters of phage bodies   Colonies, whether intracellular or discharged and persisting, showed a well-defined membrane

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ANTIBIOTICS FROM PENICILLIA

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MANY penicillia, aspergilli and basidiomycetes have been shown to produce antibacterial substances when grown under suitable conditions A summary of the information produced by surveys in this field will shortly be published (Florey, Chain, Heatley, Jennings, Sanders, Abraham, Florey, 1949) This present paper records some results of a systematic attempt to fill in gaps in our knowledge of antibiotics known to be produced by fungi, but so far not further investigated It is interesting in this respect to note that a high proportion of the penicillia examined produced already well known substances

EXPERIMENTAL

General conditions of culture

All the penicillia investigated were grown in glass vessels on 500 ml of medium 2 cm in depth Incubation was carried out at 24° C, the activity developing during culture being followed by the cylinder plate method of assay (Heatley, 1944)

Phoenicin from Penicillium rubrum

The culture fluid of *P rubrum* was reported to inhibit the growth of *Staph aureus* by Miss M E Johns

Conditions of culture

The organism was grown on glucose Sabouraud medium containing 1 per cent peptone, 2 6 per cent malt extract and 4 per cent glucose for eight days

Isolation

The culture fluid at the time of harvesting was a reddish-purple colour On acidification this colour disappeared, and the active principle was completely extracted from the acid solution into chloroform by shaking successively with one-half, one-quarter and one-eighth volumes of the solvent The yellow chloroform solution was shaken three times with one-tenth of its volume of sodium bicarbonate solution, when the active principle passed to the aqueous phase which became deep violet in colour

The extraction into chloroform and re-extraction with sodium bicarbonate was repeated, using smaller volumes of solvent, in order to effect some purification Final purification was effected by alumina chromatography   The final chloroform solution was evaporated, and the resulting yellow-brown crystalline material was recrystallized from ethanol

Chemical properties

The active substance crystallized in plates, m p 229–230° C   Found   C 58 61, H 4 17, $CH_3(C)$, 9 7 per cent, active H 1 03 per cent $C_{14}H_{10}O_6$ requires C 60 5, H 4 02, $CH_3(C)$ 10 9 per cent   It was slightly soluble in water, but soluble in chloroform, acetic acid and warm ethanol

In aqueous solution it liberated iodine from acid potassium iodide, and it was reduced to a colourless compound by sodium hydrosulphite

The substance appeared to be identical with the diquinone phoenicin, a compound isolated from *P phoenicium* v Beyma by Friedheim (1938) and examined by Posternak (1938)   Phoenicin was reported to have the molecular formula $C_{14}H_{10}O_6$ and to melt at 230° C

Antagonistic properties

The crude culture fluid showed activity against *Staph aureus*, *C xerosis* and *Myco phlei*

Only a few milligrammes of the antibiotic were available for examination At a dilution of 1 1000 it gave a zone of inhibition 22 mm in diameter when tested by the cylinder plate method against *Staph aureus*

Expansine from Penicillium equinum

The culture fluid of *P equinum* was reported to inhibit the growth of *Staph aureus* and *Bact coli* by Miss M E Johns

Conditions of culture

The fungus was grown on modified Czapek-Dox medium containing 0 3 per cent sodium nitrate, 0 1 per cent potassium dihydrogen phosphate, 0 05 per cent potassium chloride, 0 05 per cent magnesium sulphate $MgSO_4 7H_2O$, 0 001 per cent of hydrated ferrous sulphate $FeSO_4 7H_2O$ and 4 per cent of glucose   Activity was maximal after about 10–15 days

Isolation

The culture fluid was shaken with 2½ per cent acid charcoal and the active principle eluted from the latter with acetone   After distilling off most of the acetone the remaining fluid was passed through a short alumina column (pH 5), which removed much of the pigment but none of the activity   The antibiotic was extracted from the percolate with ether, and on evaporation of the ether an impure crystalline mass remained which was recrystallized from hot benzene or chloroform giving pure colourless crystals

Chemical properties

After two recrystallizations the crystals melted at 111° C On admixture with a sample of expansine (m p 111° C) the m p of the mixture was 111° C

When a solution of the crystalline material was warmed with 0 1 N sodium hydroxide solution a bright yellow colour developed After neutralizing the alkaline solution the addition of aqueous ferrous chloride gave a deep purple-red coloration The original aqueous solution gave no colour with ferric chloride solution

The substance gave a yellow precipitate with 2 4-dinitrophenylhydrazine in aqueous 2 N-hydrochloric acid which after recrystallization from methyl alcohol melted at 216° C (decomp) The corresponding product from an authentic sample of expansine melted at 216° C The substance gave an orange precipitate with phenylhydrazine hydrochloride which after recrystallization from hot water melted at 152° C The corresponding product from expansine had the same melting point (Raistrick, Birkinshaw, Bracken and Michael, 1943)

Expansine from Penicillium novae zeelandiae

The culture fluid of *P novae zeelandiae* was reported to inhibit the growth of *Staph aureus* and *Bact coli* by Miss M E Johns

Conditions of culture

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone 2 4 per cent malt extract and 4 per cent glucose Activity was maximal at about the tenth day

Isolation

The active principle was removed from the culture fluid at pH 4 by continuous extraction with ether On evaporation of the ether impure crystals were obtained These were purified by recrystallization from hot benzene

Chemical properties

The substance melted at 110° C The mixed melting point with a sample of expansine of m p 111° C was 110°C On warming a solution of the substance with 0 1 N sodium hydroxide solution a bright yellow colour developed After neutralizing the alkaline solution addition of aqueous ferric chloride gave a deep purple-red coloration The original aqueous solution gave no colour with ferric chloride solution With phenyl-hydrazine hydrochloride the substance gave an orange precipitate, which after crystallization from hot water melted at 152° C With respect to all these properties the substance was identical with expansine

Mycophenolic Acid from Penicillium viridicatum

The culture fluid of *P viridicatum* was reported by Miss M E Johns to inhibit the growth of *Staph aureus*

Conditions of culture

The fungus was grown on the modified Czapek-Dox medium used for *P equinum* containing 5 per cent neutralized corn steep liquor   Activity was maximal at about the seventh to the tenth day

Isolation

The active substance was removed from the acidified culture fluid by continuous extraction with ether   The brown gum which remained on evaporating the ether was dissolved in ethanol, and the potassium salt of the substance was precipitated by the addition of ethanolic potassium hydroxide   After centrifugation the precipitate was washed with ethanol, dissolved in water, and the free acid then precipitated by the addition of 2 N sulphuric acid   The precipitate was recrystallized from boiling water, giving small needle crystals

Chemical properties

The compound melted at 141° C   On admixture with an authentic sample of mycophenolic acid no depression in melting point was observed

The acetyl derivative was formed when 0 25 g substance was refluxed in an oil-bath at 120° C with 2 g acetic anhydride and 1 g anhydrous sodium acetate The mixture was poured into water, when the derivative formed an insoluble precipitate which was recrystallized from glacial acetic acid   M p 161° C   Mixed melting point with authentic acetyl mycophenolic acid 161° C   The compound gave a blue violet colour with ferric chloride in aqueous solution and a blue colour in alcohol

Mycophenolic Acid from Penicillium bialowiezense

The culture fluid of *P bialowiezense* was reported by Miss M E Johns to inhibit the growth of *Staph aureus*

Conditions of culture

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone   2 6 per cent malt extract and 4 per cent glucose   Activity was maximal between the twentieth and twenty-fifth days, when the culture fluid was harvested

Isolation

The culture fluid contained a penicillin-like antibiotic which was destroyed by incubation of the culture fluid for two hours at 37° C with penicillinase   The presence of this substance presumably a penicillin, was responsible for about a third of the total activity   After incubation with penicillinase the culture fluid was extracted with ether at pH 3, which removed the residual active substance The ethereal solution was concentrated and washed three times with one twentieth of its volume of saturated sodium bicarbonate solution   The main bulk of the active principle was extracted into 0 5 N sodium hydroxide solution   On addition

of hydrochloric acid to the resultant aqueous solution a white precipitate formed, which crystallized readily on scratching the vessel with a glass rod. The substance was recrystallized from hot benzene, hot water, or preferably from 1 per cent aqueous alcohol when small colourless needles were obtained.

Chemical properties

The compound melted at 141° C and on admixture with an authentic sample of mycophenolic acid no depression of m p was observed. Found C 63 7, 64 0, H 6 04 6 6 $CH_3(C)$ 7 1 per cent OCH_3 7 95 per cent. Active H 0 16 per cent M W (Camphor) 354 359, 360 (Barger) 314 $C_{17}H_{20}O_6$ requires C 63 7, H 6 3, M W 320

The acetyl derivative was prepared as in the previous example and melted at 161° C. Upon admixture with an authentic sample of acetyl mycophenolic acid no depression in m p was observed. Found C 62 5, H 6 13 $C_{19}H_{23}O_7$ requires C 62 4, H 6 07. Barer Cole and Thompson (1949) reported that the infra-red spectrum of the antibiotic isolated was identical with that of the authentic sample of mycophenolic acid

Penicillic Acid from Penicillium baarnense

The culture fluid of *P baarnense* was reported by Miss M E Johns to inhibit the growth of *Staph aureus*

Conditions of culture

The fungus was grown on a modified Czapek-Dox medium, as used for *P equinum*, together with 5 per cent neutralized corn steep liquor. Activity was maximal in 10 to 15 days

Isolation

Incubation with penicillinase destroyed about one third of the total activity of the culture fluid. The activity of the residual material was not appreciably diminished in the presence of catalase

After incubation with penicillinase, continuous extraction of the culture fluid at pH 2 with ether and evaporation of the ether yielded an oily material from which impure orange-coloured crystals were obtained

The material was passed through an acid-washed alumina column (pH 5), when an active fraction was obtained which yielded almost colourless crystals

Chemical properties

The crystalline material was recrystallized from hot water, yielding crystals melting at 65° C. After recrystallization from petroleum ether the substance melted at 84° C. It was found that specimens of penicillic acid from *P baarnense*, *A quercinus* and *A melleus*, partly melted at 38° C when crystallized from water, ethanol or ether. The low-melting compound was a hydrate which was dehydrated in a vacuum desiccator over phosphorus pentoxide to give the non-hydrated penicillic acid m p 84° C. Found C 56 4, H 6 4 $C_8H_{10}O_4$ requires C 56 4 per cent, H 5 89 per cent. With ammonium hydroxide

a marked pink colour characteristic of penicillic acid developed, and upon admixture of the crystals with an authentic sample of penicillic acid no depression in melting point occurred

With phenylhydrazine the substance gave a yellow crystalline derivative, m p 171° C

The corresponding derivative from penicillic acid has been reported to melt at 171° C and 175° C (Alsberg and Black, 1913 , Birkinshaw, Oxford and Raistrick, 1936)

Herquein from Penicillium herquei

The culture fluid of P herquei was reported by Miss M E Johns to inhibit the growth of Staph aureus   The antibacterial substance in the fluid has been isolated and named herquein

Conditions of culture

The fungus was grown on a modified Czapek-Dox medium, as used for P equinum

Activity appeared between the 8th and 15th days and disappeared within 24 h ns   Frequent examination of the culture fluid and rapid extraction of the active principle when produced were essential for the successful isolation of the antibiotic

Isolation

The culture fluid was acidified to pH 2 when a precipitate formed carrying with it all the activity   The precipitate was centrifuged, suspended in water and extracted with chloroform   The active principle was extracted from the chloroform into aqueous solution at pH 7 5, and was then precipitated as a yellow microcrystalline solid by addition of hydrochloric acid   It was recrystallized from aqueous alcohol or benzene

Chemical properties

Herquein forms yellowish-brown crystals, m p 129° C (decomp)   Found C 59 84 H 5 42, OCH$_3$ 5 33, CH$_3$(C) 10 5 per cent   C$_{19}$H$_{20}$O$_8$ requires C 60 6, H 5 2   The substance is sparingly soluble in water, giving a yellow solution which exhibits a bottle-green fluorescence on addition of alkali

It is readily soluble in ethanol and chloroform moderately soluble in ether and carbon tetrachloride slightly soluble in benzene and insoluble in petroleum ether   It can be precipitated from aqueous solution by means of lead acetate, and recovered by decomposing the lead with sodium sulphate

Antibacterial properties

Di J S Robertson tested the crude culture fluid against a wide variety of organisms, and reported activity against Sh shigae, Myco phlei, Str pyogenes, V cholerae Staph albus, Staph aureus and Ps pyocyanea

Tested by the wheel plate method, a 1/500 solution of herquein inhibited the growth of the El Tor vibrio, V cholerae, C xerosis and Staph aureus

In serial dilution tests it inhibited the growth of Staph aureus and V cholerae at 1 2500, but not that of the El Tor vibrio

Penicillin like Antibiotics from Penicillia

The culture fluids of P euglaucum P meleagrinum, P divaricatum, P roseo-citreum Biourge (Baarn), P roseo citreum Biourge (N C T C) and P roseo-citreum (P 94) were all reported to inhibit the growth of Staph aureus by Miss M F Johns

Conditions of culture

P euglaucum was grown on a modified Czapek-Dox medium, as used for P equinum, together with 5 per cent neutralized corn steep liquor Activity was maximal in about 10 to 15 days

P meleagrinum was grown on the above medium Activity was maximal in 5 to 10 days

P roseo-citreum (N C T C strain) and P roseo-citreum (P 94) were grown on the above medium whilst P roseo-citreum (Baarn) was grown using a similar medium but without the addition of corn steep liquor Activity was maximal in 10 days

Extraction

In all these cases the culture fluid was acidified to pH 2 with hydrochloric acid, and extracted with one seventh volume of either ether or ethyl or amyl acetates All the activity was extracted from the culture fluid at pH 2 and none at pH 7 or pH 10 The organic solvent layer was extracted with phosphate buffer at pH 7 when the activity passed entirely into the aqueous layer

Chemical properties

In each case activity was totally destroyed by incubation of either the crude culture medium or the extract for two hours at 37° C with penicillinase or by incubation in the presence of copper sulphate

The activity was also destroyed by heating the extract at 100° C for 15 minutes at pH 2 and at pH 9, and was partially destroyed by heating for 15 minutes at pH 7

It is concluded that these penicillia produce penicillin-like substances

SUMMARY

Antibacterial substances have been extracted from the culture fluids of 13 penicillia

It has been found that six of these, P meleagrinum Biourge, P euglaucum, P divaricatum and three strains of P roseo-citreum produce penicillin-like antibiotics alone, and that two others, P baarnense v Beyma and P bialowiezense Zal, produce a penicillin-like antibiotic, together with other antibiotics P baarnense produces penicillic acid, P bialowiezense and P viridicatum produce mycophenolic acid, and P equinum v Beyma and P novae zeelandiae v Beyma produce expansine P herquei N C T C 1721 produces an antibiotic named herquein, which inhibits the growth of Staph aureus and some vibrios at high concentrations P rubrum produces a compound which has antibacterial properties which has been shown to be phoenicin

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SURVEY OF PAPERS

GOTTSCHALK AND LIND find that ovomucin is the only component of hen egg-white that has the power to inhibit haemagglutination by heated Type B influenza virus. The inhibitory power of ovomucin is readily reduced by active influenza virus Types A and B by the receptor-destroying enzyme of the *Vibrio cholerae* and also by trypsin (p. 85)

LINGGOOD AND WONWON have confirmed with two dimensional paper partition chromatography their original observations regarding the utilization of various amino acids from casein hydrolysate medium during diphtheria toxin production with their strain of PW 8 and on the basis of these results have used a modified medium fortified with glutamic acid instead of glycine, which, in their hands gives high titre toxin (100 Lf units/ml.) more consistently and is accompanied by a more complete utilization of amino acids as detected by paper chromatography (p. 93)

BARNES has investigated an antibiotic produced by a strain of *B. laterosporus*. Evidence is presented for the existence of two separate though closely related substances which have been named laterosporin A and laterosporin B (p. 100)

GOVAN AND PARKIN find that the oral administration of calcium chloride to rabbits causes extensive focal necrosis in the liver and necrosis of the tubules in the juxta medullary region of the kidney (p. 105)

GILL-CAREY has examined 37 species of aspergilli for the ability to produce antibiotics and finds that 20 strains produce substances with some antibacterial activity (p. 114)

GILL CAREY has made a further investigation of the antibiotics produced by one strain each of six species of the aspergilli described in the preceding paper (p. 119)

HOYLE describes a method of observing the effect of any substance on the intracellular growth of influenza virus A in egg cultures. He finds that certain triphenylmethane dyes can readily retard and reduce the intracellular growth of the virus (p. 123)

GARDNER describes the production and properties of an antibiotic formed by a strain of *Staph aureus*. The properties of the antibiotic are such as to make it probable that it will have no thera peutic application (p. 130)

MERLING has made an electron microscopical study of the infection of *Bact. coli* by bacteriophage Bacteriophage appears to multiply by binary fission which is seen either in freshly infected cells a few minutes after entry or in discharged clusters of phage bodies (p. 139)

STOWAR BURTON describes the production and properties of antibacterial substances which have been extracted from the culture fluids of 13 penicillia (p. 151)

(1)

THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY PART IV A SURVEY OF HETEROCYCLIC BASES, WITH SPECIAL REFERENCE TO BENZQUINOLINES, PHENANTHRIDINES, BENZACRIDINES, QUINOLINES AND PYRIDINES

A ALBERT, S D RUBBO and MARGARET I BURVILL

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A CLOSE correlation between kationic ionization and antibacterial properties has been demonstrated in the acridine series (Albert, Rubbo, Goldacre, Davey and Stone, 1945, Albert and Goldacre, 1948) Those acridines which are sufficiently strong bases to be more than 50 per cent ionized at pH 7 3 were found to exert a strong bacteriostatic action, whereas weaker bases were found relatively ineffective Because no other series of bases has ever been investigated along these lines, it was not known whether this type of correlation pertains only to the acridine series or is capable of wider application

The aim of the present work has been to extend this investigation to a representative number of other heterocyclic series in order to discover whether these would resemble the acridines in that the highly ionized members would be more antibacterial than the poorly ionized members

The results of these investigations plainly showed that kationic ionization markedly increases antibacterial action in the following seven series, the three isomeric benzquinoline series, the phenanthridines, and the three isomeric benzacridine series

All these substances have molecules with a large area of flat surface—an architectural feature which was shown to be necessary for this type of antibacterial action When it was incorporated in the quinoline and the pyridine series, the correlation between ionization and antibacterial properties was again easily demonstrated These results led to a general discussion of the factors which govern the antibacterial action of organic bases

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MATERIALS AND METHODS

Chemical

Organic chemistry

Of the 96 substances listed in Tables I, II, III, IV, V and VII, 33 were previously unknown, and had to be specially synthesized to fill important gaps in the data   The preparation of the new benzquinolines, phenanthridines and benzacridines is described by Albert, Brown and Duewell (1948), and of the older examples by Albert, Goldacre and Phillips (1948)   The three series of benzquinolines (II, III and IV, Table I), the phenanthridines (V) and the three series of benzacridines (VI, VII and VIII, Table II) have been numbered according to the British system, those who use the American system will find a key for inter-conversion in Table VIII

The preparation of the styryl compounds, the guanidines and the diguanides has been described by Royer (1949)   The acridine derivatives were described in our previous paper (Albert *et al*, 1945)   The aliphatic amines (No 87–89) in Table VII were kindly supplied by Dr K Sutherland, of the Division of Chemistry, Council for Scientific and Industrial Research, Melbourne   The source of the remainder of the substances is as given by Albert, Goldacre and Phillips (1948), except where otherwise indicated

Careful attention was paid to bringing all test-substances to a state of complete analytical purity

Physical chemistry

The majority of the pK_a values given in this paper have been taken from Albert Goldacre and Phillips (1948)   The others, unless otherwise mentioned in footnotes to the tables, were kindly determined by Mr J N Phillips

Some of the published pK_a values have been determined only in dilute alcohol, so that it was necessary to add a correction to convert these figures to the equivalent values for water   For each series the magnitude has been obtained by averaging those corrections found necessary where pK_a values have been determined both in water and alcohol, as follows

Benzquinolines and phenanthridines	.	1 0 unit
Benzacridines		0 7 ,,
Acridines, quinolines and pyridines		0 5 ,,

Those values which have been obtained by correction have been placed in parentheses in the tables   They are subject to an error of about $\pm$ 0 2 unit, which is of no consequence in the present work except for pK_a values lying between 6 3 and 8 3, at pH 7 3, where a small change in pK_a can cause a relatively large change in percentage ionization because of the shape of the curve at this point   Fortunately very few values falling within this range had to be corrected

Bacteriological

The technique, the test organisms and the medium (Wright's heart broth with added peptone and 10 per cent of sterile, unheated ox serum) were as defined by Albert *et al* (1945)   The usual heavy inoculum was used, viz 0 03 ml of a 24-hour culture in 2 5 ml of medium, thus providing at least one million organisms per tube

The biological results in the tables are recorded in terms of the actual chemical *base* in order to eliminate errors due (i) to varying amounts of water of crystallization and (ii) to acid radicals of different molecular weight The free bases were dissolved in water with the aid of 1 to 1 5 equivalents of hydrochloric or acetic acid

That dilution completely preventing visible growth after 48 hours' incubation at 37° C was taken as the end-point of bacteriostasis To this dilution a code number was assigned according to the following key

Key to dilutions to be used for all tables dealing with antibacterial activity

0 signifies growth at 1 in 5000 (calculated as base)
1   ,,   inhibition of growth at 1 in   5000
2   ,,   ,,   ,,   1 ,,   10,000
3   ,,   ,,   ,,   1 ,,   20,000
4   ,,   ,,   ,,   1 ,,   40,000
5   ,,   ,   ,,   1 ,,   80,000
6   ,,   ,,   ,,   1 ,, 160,000
7   ,,   ,,   ,,   1 ,, 320,000
8   ,,   ,,   ,,   1 ,, 640,000

It will be appreciated that, as these figures vary by a factor of two, a substance giving the reading " 6 " is eight times (2^3) as powerful as one giving the reading " 3 "

All tests were done in duplicate In the case of all substances having special bearing on the hypotheses developed here, the tests were repeated, again in duplicate

RESULTS

(1) *The Isomerides of Acridine*

Acridine may be regarded as 2 3-benzquinoline It was therefore decided to examine all the isomerides arising when one benzene ring in acridine is moved
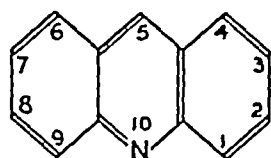
(I, Acridine)   (II, 5 6-Benzquinoline)   (III, 6 7-Benzquinoline)
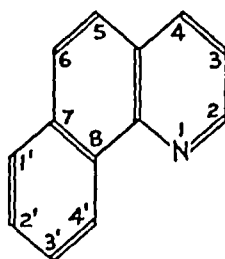
(IV, 7 8-Benzquinoline)   (V, Phenanthridine)
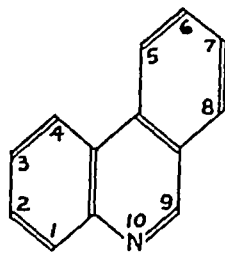
TABLE I—*Antibacterial Properties of Benzquinolines and Phenanthridines Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C*

Medium   10 per cent serum broth, pH 7 2–7 4

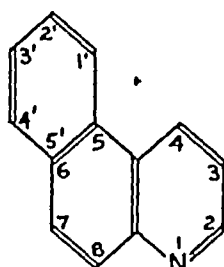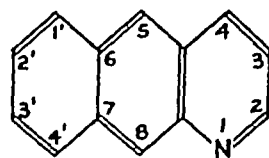| No	Substance	Cl welchii	Str pyogenes	Staph aureus	B coli	Proteus	Bacterio static index (sum total of code numbers of inhibitory dilutions)	pKa in water at 20°C	Per cent ionized (kation) at 20°C, pH 7 3
1.	5 *Aminoacridine* (for comparison)	6	6	4	4	4	24	10 0	100
	5 6-*Benzquinoline*—								
2	(Unsubstituted)	1	1	1	0	0	3	5 1	<1
3	1′ Amino-	2	0	0	0	0	2	5 0	<1
4	3′-Amino-	0	0	0	0	0	0	(5 0)	<1
5	4′-Amino-	1	1	0	0	0	2	5 2	<1
6	2-Methyl-	2	1	1	0	0	4	(5 4)	1
7	2′ 4′-Diamino-	0	0	0	0	0	0	(5 9)	4
8	2-Amino-4 methyl-	4	3	0	0	0	7	7 1	39
9	4-Amino-	6	3	2	3	2	16	(9 0)	98
10	4 Amino-2 methyl-	5	4	2	3	1	15	(9 4)	99
	6 7-*Benzquinoline*—								
11	(Unsubstituted)	1	2	0	0	0	3	5 0	<1
12	8 Chloro-	0	0	0	0	0	0	(3 5)	<1
13	4-Amino 2-methyl 8 chloro-	2	2	0	0	0	4	(6 9)	28
14	3 4-Diamino-	5	4	2	2	0	13	(9 1)	98
15	4-Amino-	7	4	3	3	2	19	(9 7)	100
16	4 Amino-2-methyl-	6	5	3	3	1	18	(10 4)	100
	7 8 *Benzquinoline*—								
17	(Unsubstituted)	2	0	0	0	0	2	4 2	<1
18	1′-Amino 2 methyl-	0	0	0	0	0	0	(5 7)	3
19	6 Amino-2 methyl-	0	0	0	0	0	0	(6 2)	7
20	2 Amino 4 methyl-	2	0	0	0	0	2	6 7	20
21	4 Amino	5	5	3	4	2	19	(8 7)	96
22	4-Amino 2-methyl-	6	5	3	3	1	18	(9 0)	98
	Phenanthridine—								
23	(Unsubstituted)	2	2	1	0	0	5	(4 3)	<1
24	7 Amino 9 methyl-	0	0	0	0	0	0	(6 2)	7
25	2 Amino 9 methyl-	3	2	1	0	0	6	(6 7)	20
26	9 Amino-	4	4	2	2	2	14	7 3	50
27	2 7 Diamino 9 methyl-	5	4	3	1	0	13	(7 3)	50
28	2 7 9 Triamino	6	5	4	1	0	16	(9 1)	98

to all the other possible positions on the quinoline skeleton   Thus 3 4-benzquinoline (which is known as "phenanthridine"), and the 5 6-, 6 7- and 7 8-benzquinolines, which do not have special names, were examined   To these were added a sufficient number of their amino-derivatives, so chosen as to cover the range from the almost non-ionized to the almost completely ionized at the pH of the test, viz pH 7 3   The results are given in Table I, and the relevant structural formulae are at the head of this table   5-Aminoacridine (Aminacrine, B P), which is one of the best of the highly ionized acridines, has been included as a standard

It is seen from Table I that a strong positive correlation exists, in all four series, between the ionization of the bases and their antibacterial action   As

soon as the basic strength is high enough to bring about ionization to the extent of 50 per cent or more, the antibacterial titre increases considerably In short, the bacteriostatic index remains below 7 as long as the ionization remains below 50 per cent , but when the ionization equals or exceeds this figure, scores as high as 19 (e g 4-amino-7 8-benzquinoline, No 21) are obtained

Although this correlation resembles that found in the acridine series, it is evident that the highest score, 19, falls short of the figure, 24, obtained with 5-aminoacridine (No 1), and that the four series exemplified in Table I are not all equally active

(ii) The Benzologues of Acridine

Benzologues are those substances differing from one another by the presence of more, or fewer, benzene rings, each of which shares two carbon atoms with
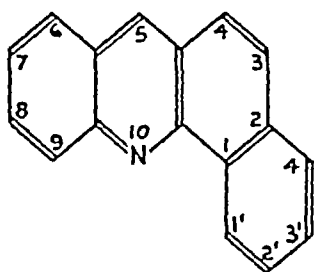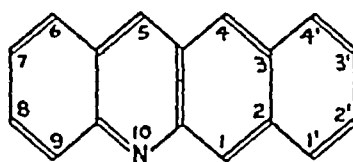
(VI, 1 2-Benzacridine)   (VII, 2 3-Benzacridine)   (VIII, 3 4-Benzacridine)
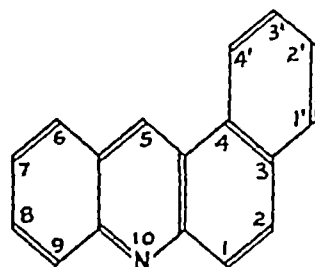
TABLE II —Antibacterial Properties of Benzacridines   Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C
Medium   10 per cent serum broth, pH 7 2–7 4

No	Substance	Cl welchii	Str pyogenes	Staph aureus	B coli	Pro teus	Bacteriostatic Index (sum total of code numbers of inhibitory dilutions)	pKa in water at 20° C	Per cent ionized (kation) at 20° C, pH 7 3
	1 2 Benzacridine—								
29	(Unsubstituted)	0	0	0	0	0	0	(4 1)	<1
30	7-Amino-	0	0	0	0	0	0	(4 7)	<1
31	8 Amino	4	4	3	0	0	11	6 7	20
32	5 Amino 4' nitro-	6	1	0	0	0	7	(7 1)	39
33	5 Amino-	8	6	6	3	0	23	(8 8)	97
34	4' 5 Diamino-	7	7	6	3	2	25	(9 1)	98
	2 3 Benzacridine—								
35	(Unsubstituted)	1	2	0	0	0	3	(5 2)	<1
36	5 Acetamido	6	0	0	0	0	6	(5 3)	1
37	7 Amino-	2	0	0	0	0	2	(6 1)	6
38	5 Amino	7	7	5	4	1	24	(10 4)	100
	3 4 Benzacridine—								
39	(Unsubstituted)	0	0	0	0	0	0	4 7	<1
40	8-Acetamido-	0	0	0	0	0	0	(5 2)	<1
41	7 Amino	0	0	0	0	0	0	(5 7)	3
42	8 Dimethylamino-	4	5	2	0	0	11	7 3	50
43	8 Amino	7	5	5	0	0	17	7 4	56
44	5 Amino-	7	6	5	3	2	23	(9 1)	98

another ring   The most familiar example of benzologues is the family   benzene, naphthalene and anthracene   Pyridine, quinoline, acridine and the benzacridines form a similar family of benzologues, the members of which have now been examined for evidence of correlation between ionization and antibacterial action (Tables II and III)   A sufficient number of amino-derivatives have been included to cover a wide range of percentage ionization, and, in the case of the aminoquinolines and aminopyridines, every possible isomeride has been tested

It is seen from Table II that a strong positive correlation exists, in all three benzacridine series, between the strength of the bases and their antibacterial action   Those bases strong enough to be more than 50 per cent ionized are very much more antibacterial than those which fall below this figure   The high scores in this series (e g a bacteriostatic index of 23 to 25 for No 33, 34, 38 and 44) show it to be the equal of the acridine series (cf 5-aminoacridine, No 1, one of the best of the amino-acridines)

(IX, Quinoline)   (X, Pyridine)

TABLE III —Antibacterial Properties of Quinolines and Pyridines   Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C
Medium   10 per cent serum broth, pH 7 2–7 4

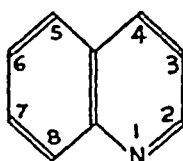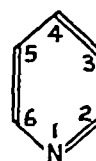| No	Substance	Organisms					Bacteriostatic index (sum total of code numbers of inhibitory dilutions)	pKa in water at 20° C	Per cent ionized (kation) at 20° C , pH 7 3
		Cl wel chu	Str pyo genes	Staph aur eus	B coli	Pro- teus			
	Quinoline—								
45	(Unsubstituted)	0	0	0	0	0	0	4 9	<1
46	8 Amino	0	0	0	0	0	0	4 0	<1
47	3 Amino-	0	0	0	0	0	0	4 9	<1
48	5 Amino	0	0	0	0	0	0	5 5	2
49	6 Amino-	0	0	0	0	0	0	5 6	2
50	7 Amino	0	0	0	0	0	0	6 6	17
51	2-Amino	0	0	0	0	0	0	7 3	50
52	4 Amino	0	0	0	0	0	0	9 1	98
53	2 4 Diamino	2	2	0	0	0	4	9 4	99
54	4 Amino 2 methyl-	0	0	0	0	0	0	9 4	99
55	4 Amino 2 6 8 trimethyl-	2	2	0	1	0	5	10 4	100
56	4-Amino 2 phenyl-	3	2	1	1	0	7	(8 2)	89
	Pyridine—								
57	(Unsubstituted)	0	0	0	0	0	0	5 2	<1
58	3 Amino-	0	0	0	0	0	0	6 0	5
59	2 Amino-	0	0	0	0	0	0	6 9	28
60	4-Amino-	0	0	0	0	0	0	9 1	98

It is seen from Table III that the quinolines and pyridines, which are the lower benzologues of the acridines, are almost devoid of antibacterial action, even when well ionized   Their lack of antibacterial properties can be explained
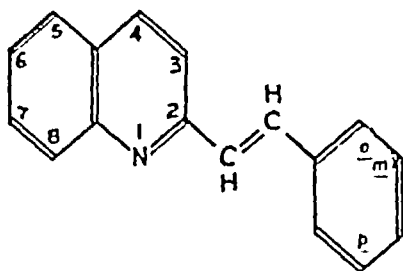
(XI 2-Styrylquinoline)

(XII, 2-(δ-Phenylbutadienyl)-quinoline)
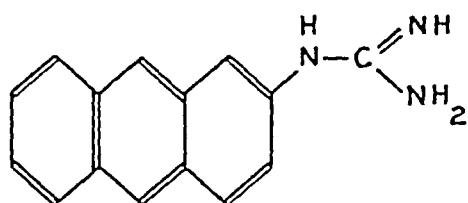
(XIII, 2-Anthrylguanidine)
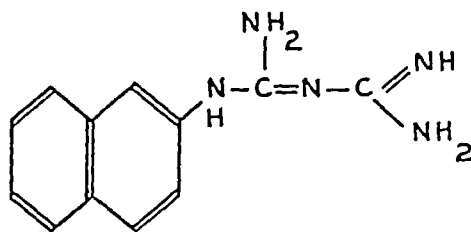
(XIV, 2-Naphthyldiguanide)

TABLE IV —*The Effect upon Antibacterial Action of Decreasing or Increasing the Flat Area of Various Molecules Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C*
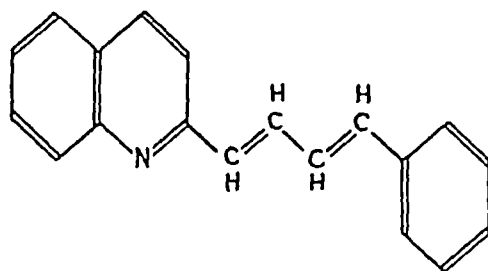
Medium 10 per cent serum broth, pH 7 2–7 4

No	Substance	Organisms					Bacterio-static index (sum total of code numbers of inhibitory dilutions)	pKa in water at 20° C	Per cent ionized (kation) at 20° C, pH 7 3
		Cl wel chu	Str pyo- genes	Staph aur- eus	B coli	Pro teus			
A	*Area diminished—*								
61	5 Amino 6 7 8 9 tetrahydro-acridine	0	1	0	0	0	1	(9 9)	100
(1)	(5 Aminoacridine for comparison)	6	6	4	4	4	24	10 0	100
62	5-Amino-6 7 8 9 tetrahydro 2 3-benzacridine	3	4	4	2	0	13	(10 3)	100
(38)	(5-Amino 2 3 benzacridine for comparison)	7	7	5	4	1	24	(10 4)	100
B	*Area increased—*								
63	6 Amino 2 styrylquinoline	0	0	0	0	0	0	(5 5)	2
(49)	(6 Aminoquinoline for comparison)	0	0	0	0	0	0	5 6	2
64	4-Amino 2 styrylquinoline	5	5	4	2	0	16	(9 3)	99
(52)	(4-Aminoquinoline for comparison)	0	0	0	0	0	0	9 1	98
65	4-Amino 2 (p aminostyryl) quinoline	5	5	2	3	0	15	ca 9 5	99
66	4-Amino 2 (p dimethylamino styryl) quinoline	4	4	4	2	0	14	(9 7)	99
67	4-Amino 6-ethoxy-2 styryl-quinoline	5	6	4	0	0	15	ca 9 5	99
68	4 Amino 6 ethoxy 2 (δ-phenyl butadienyl)quinoline (XII)	6	6	3	0	0	15	ca 9 5	99
69	4 Amino-2 6 distyrylpyridine	5	5	3	1	0	14	(8 6)	95
(60)	(4 Aminopyridine for comparison)	0	0	0	0	0	0	9 1	98
C	*Series of increasing area—*								
70	Phenylguanidine*	0	0	0	0	0	0	10 9‡	100
71	2 Naphthylguanidine	0	0	0	0	0	0	10 7	100
72	2-Anthrylguanidine (XIII)	4	4	4	0	0	12	11 0	100
73	Phenyl-N¹ diguanide†	0	0	0	0	0	0	10 7	100
74.	2-Naphthyl N¹ diguanide† (XIV)	1	1	0	0	0	2	10 7	100
75	2-Anthryl N¹ diguanide	4	4	4	0	0	12	10 4	100

* Prepared as in Smith (1929)
† Prepared as in Cohn (1911) and Smolka and Halla (1901)
‡ From Davis and Elderfield (1932)

by the decreased area of the surface of these molecules   It has been noted (Albert *et al* , 1945) that a highly significant decrease in antibacterial properties follows the hydrogenation of 5-aminoacridine to 5-aminotetrahydroacridine, an operation which reduces the flat area of the parent molecule   This important phenomenon has been confirmed, as shown in Table IV, where the activities of 5-aminoacridine (No 1) and 5-aminotetrahydroacridine (No 61) are compared An attempt was made to restore antibacterial action to the quinoline, pyridine and tetrahydroacridine series by increasing the area of the flat surface of their molecules in various ways, which will now be described   These modifications of structure will be seen to have caused a profound increase in antibacterial activity

It can be seen from Table IV that the addition of a styryl group to 4-amino-quinoline (No 52), which is not antibacterial, gives 4-amino-2-styrylquinoline (No 64), thereby introducing into the quinoline series antibacterial properties of the same order as the best of the benzquinolines   No 65, 66, 67 and 68 are further examples   Similarly, the addition of two styryl groups to 4-amino-pyridine (No 60), which is not antibacterial, gives 4-amino-2 6-distyrylpyridine (No 69), which has considerable antibacterial activity   6-Amino-2-styryl-quinoline (No 63) has been included to demonstrate that an increase in flat surface does not of itself increase antibacterial action   the base must be strong enough to secure a high degree of ionization at the pH of the test

The addition of a flat ring to 5-aminotetrahydroacridine (No 61) gave its benzologue, 5-aminotetrahydrobenzacridine (No 62)   Parallel to this restoration of flatness, a considerable proportion of the antibacterial activity is seen to have been restored

No 70–75 are two series of non-heterocyclic benzologues, the aryl-guanidines and the aryl-diguanides   It is seen from Table IV that here also a strong antibacterial action does not appear until a sufficient area of flat surface of the molecule has been built up as in the three-ring anthracene nucleus of No 72 and 75
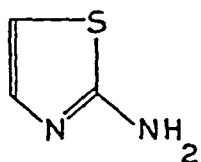
(XV, 2-Aminothiazole)
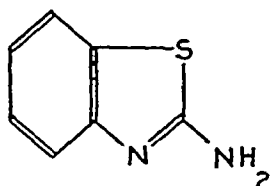
(XVI, 2-Aminobenz-
thiazole)
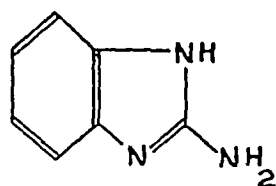
(XVII, 2-Aminobenz-
iminazole)
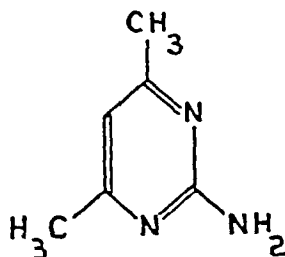
(XVIII, 2-Amino-4 6-
dimethylpyrimidine)
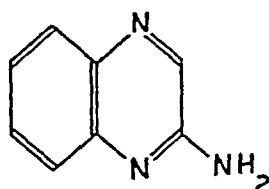
(XIX, 2-Amino-
quinoxaline)
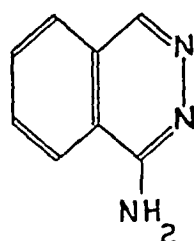
(XX, 1-Amino-
phthalazine)
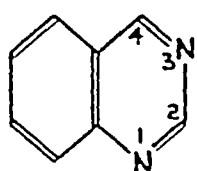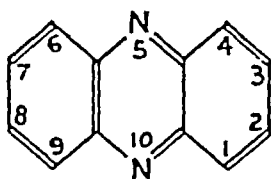
(XXI, Quinazoline)   (XXII, Phenazine)   (XXIII, Thionine)
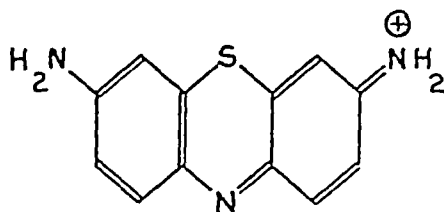
TABLE V.—*Examples of Lack of Antibacterial Activity in Heterocyclic Amines which have One or More of the Following Defects   (a) Insufficiently Ionized, (b) Insufficient Flat Area per Molecule, (c) Well Ionized, but Easily Reduced to Poorly Ionized Substance   Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C*

Medium   10 per cent serum broth, pH 7 2–7 4

| No | Substance | Type of defect | Organisms | | | | | Bacteriostatic Index (sum total of code numbers of inhibitory dilutions) | pKa in water at 20° C | Per cent Ionized (kation) at 20° C, pH 7 3 |
			Cl tret chu	Str pyo- genes	Staph aur eus	B coli	Pro- teus			
76	2-Aminothiazole (XV)	A, B	0	0	0	0	0	0	5 4	1
77	2-Aminobenzthiazole (XVI)	A B	0	0	0	0	0	0	4 5	<1
78	2-Aminobenziminazole (XVII)	B	0	0	0	0	0	0	7 5	61
79	2 Amino 4 6 dimethyl-pyrimidine (XVIII)	A, B	0	0	0	0	0	0	4 8	<1
80	2 Aminoquinoxaline (XIX)	A, B	0	0	0	0	0	0	4 0	<1
81	1-Aminophthalazine (XX)	A, B	0	0	0	0	0	0	6 6	17
82	2 Aminoquinazoline	A, B	0	0	0	0	0	0	4 4	<1
83	4 Aminoquinazoline	A, B	0	0	0	0	0	0	5 7	3
84	2 Amino 8-dimethylamino 3 methylphenazine (neutral red)	A	0	0	0	0	0	0	6 9*	28
85	Thionine (Lauth's violet) (XXIII)	C	2	1	2	0	0	5	oxidized 11† reduced 5 3†	100 1
86	Tetramethylthionine (methylene blue)	C	1	3	1	0	0	5	oxidized >11† reduced 5 8†	100 3

* Clark and Perkin (1932)
† Phillips, Clark and Cohen (1927)

A number of heterocyclic bases, belonging to nine series not previously discussed, have been sampled in Table V   It will be seen from Table V that none of these substances achieves high antibacterial activity   The reasons for this lack are dealt with further in the discussion

DISCUSSION

(1) Heterocyclic Bases

This discussion is built around the two principles which the foregoing results indicate to be the most important limiting factors governing antibacterial activity in heterocyclic bases   These are (a) adequate ionization and (b) adequate area for the flat surface of the molecules

It had been shown in Part II (Albert *et al*, 1945) that a high degree of bacteriostasis in the acridine series occurs only in those members which are strong enough bases (as measured by the pK_a value) to ionize to the extent of at least 50 per cent at pH 7 3

A similar correlation between bacteriostasis and ionization has now been demonstrated in a number of other heterocyclic series (Tables I, II and IV). The most obvious examples are those contained in Table I, which deals with the benzquinolines and phenanthridines (which are isomerides of acridine), and in Table II, which deals with benzacridines, which are higher benzologues of acridine (see illustrations at the head of tables)   In the three isomeric series of benzacridines (Table II), the maximal antibacterial activities are equal to those commonly found among well-ionized acridines (e g 5-aminoacridine, No 1 in Table I) In the other series (Table I) the highly ionized members often have much the same degree of activity as 5-aminoacridine towards Gram-positive species, but are relatively inactive towards the Gram-negative species tested here   Actually a few acridines which behave in the same way were described in Part II, e g, 2 5-diaminoacridine, 5-amino-1-phenylacridine and 2 8-*bis*dimethylaminoacridine Three benzquinolines (Nos 2, 7 and 17) were previously examined by Browning, Cohen, Gaunt and Gulbransen (1922) who found them inactive and concluded that the benzquinolines were in no way comparable with the acridines as antibacterials   It was unfortunate that their examples were all poorly ionized, although at the time neither the magnitude of the ionization constants nor the significance of ionization in bacteriostasis was known

A similar mode of action may therefore be postulated for the acridines and the seven series described in Tables I and II   This similarity does not rest solely upon the basis of adequate ionization, because a practically complete lack of antibacterial power has been demonstrated in the *lower* benzologues of acridine (quinolines and pyridines, Table III)   An apparent exception, 8-hydroxyquinoline, is antibacterial because of a peculiarity in its molecular architecture which enables it to chelate with metals (see Albert, Rubbo, Goldacre and Balfour, 1947)   Some amino-quinolines and -pyridines (excluding all the highly ionized members) had previously been examined by Browning *et al* (1922), with results similar to those reported here

A likely explanation for this low activity is forthcoming from the results reported in Part II for 5-amino-6 7 8 9-tetrahydroacridine   It will be recalled that this substance was found to be almost lacking in antibacterial properties— a fact confirmed in the course of the present work (No 61, Table IV)   As pointed out in Part II, this substance, which at first sight differs from the highly antibacterial 5-amino-acridine (No 1) only in the possession of four extra hydrogen atoms, owes its poor antibacterial properties to the loss of an important proportion of the flat surface of the molecule   Those molecules which, like 5-aminoacridine, are conjugated (i e every second bond is a double-bond) throughout are flat, virtually two-dimensional structures, hence all the atoms constituting the ring-system lie in the plane of the paper   However, any such molecule, when hydrogenated loses its planar configuration   Such an effect can be seen in Fig 1, where 5-aminoacridine and its tetrahydro-derivative are shown, built to scale with Herschfelder atomic models   It is seen from Fig 1 that the molecule of 5-aminoacridine is planar enough to lie flat on a surface, but its tetrahydro-derivative cannot do so because of the distortion brought about by hydrogenation
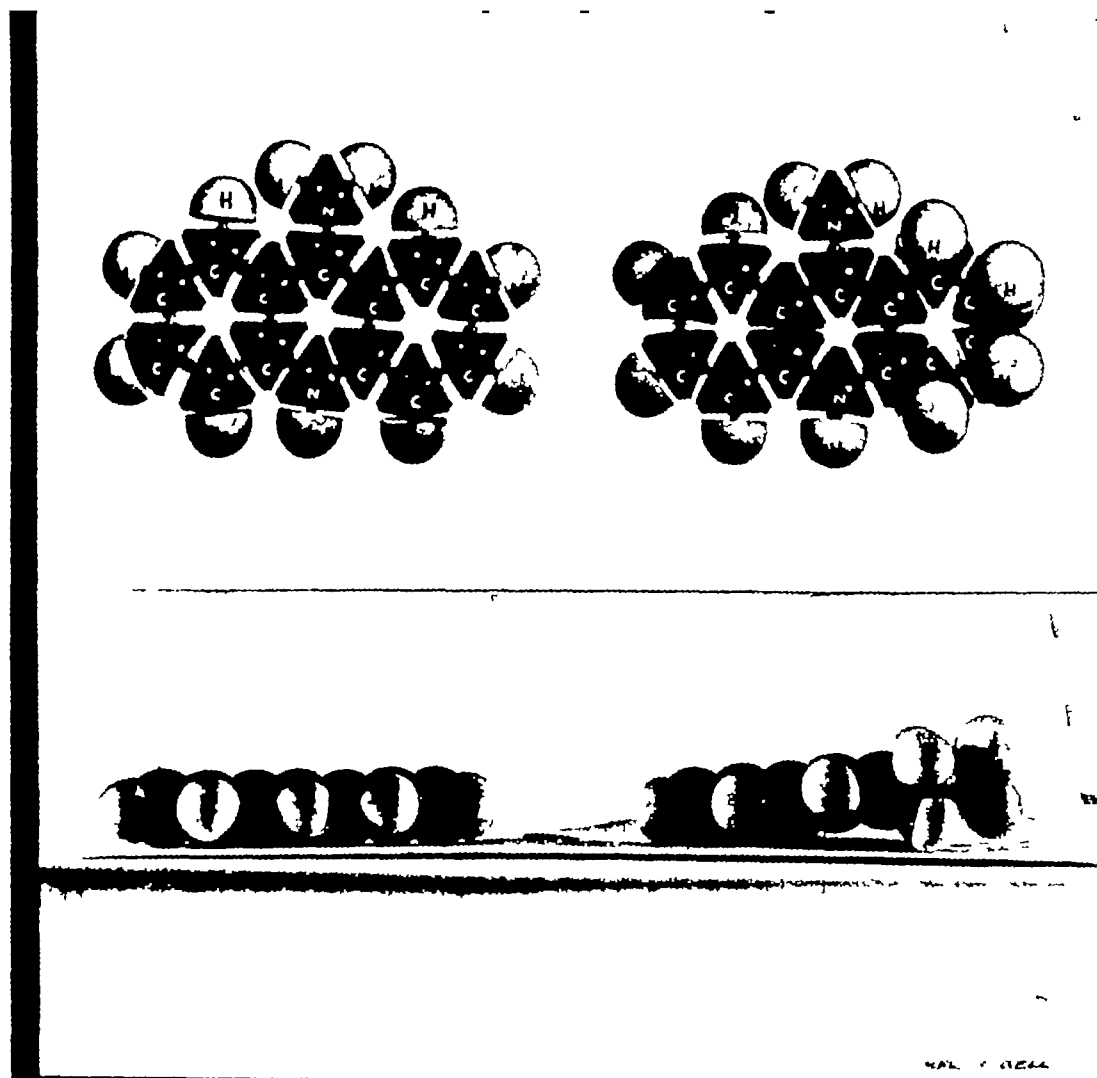
1

FIG 1 —Loss of flatness on partial hydrogenation   Scale models of the ions of 5 aminoacridine
(left hand pair) and 5 aminotetrahydroaeridine (right hand pair)

Albert, Rubbo, and Burvill

The problem of increasing the flat area of 4 aminoquinoline was solved by providing it with a (trans) styryl substituent, giving e g 4-amino-2-styryl-quinoline (No 64) It is well established in stereochemistry that the styryl-group takes up a position co-planar with the nucleus to which it is attached (cf the work of Robertson (1935) and Robertson and Woodward (1937) on trans-stilbene, i e styryl-benzene) As may be seen from Table VI, the insertion of a styryl-group has enlarged the flat area of the quinoline molecule even beyond that of the acridine molecule Naturally enough, this increase in area has no effect on the antibacterial properties when the operation is performed on a feebly ionized aminoquinoline e g when 6-aminoquinoline is converted to 6-amino-2-styrylquinoline (No 63) On the other hand, the effect of adding a styryl group to 4-aminoquinoline, which is well ionized, is to raise its antibacterial power from zero up to a figure comparable with the best of the benzquinolines, namely to a B I of 16 Further examples of this effect are seen in No 65–68 (the δ-phenylbutadienyl-group in No 68 is a higher vinylogue of the styryl group)

Similarly the addition of styryl-groups to 4-aminopyridine, giving 4-amino-2 6-distyrylpyridine (No 69), has increased the antibacterial properties from zero to a high level In assessing the activity of this substance it should be noted that the molecular weight is 298 , hence it is 65 per cent heavier than any monoamino-benzquinoline or -acridine As all the figures in this paper are reported on a weight- (as distinct from a molar-) basis, these heavier molecules (such as No 69) are even more potent than the figures suggest

These considerations of the necessity for a minimal flat area do not apply exclusively to heterocyclic molecules It is evident from Table IV that strong antibacterial activity appears in two series (the aryl-guanidines and the aryl-diguanides) as soon as the flat area of the molecule corresponds to that of the

acridine and benzquinoline molecules (as in anthrylguanidine and anthryl-diguanide, No 72 and 75)   It should be pointed out that although the guanidine and diguanide ions are themselves flat, spectrographic evidence has shown no conjugation between these groups and the aryl nuclei   Hence free-rotation, and not compulsory co-planarity, governs the relations of nucleus and side-chain in these cases

Table VI has been compiled to assist quantitative appraisal of the flat-area effect   The dimensions of the nuclei have been calculated accurately from the tables of bond-distances given by Branch and Calvin (1941)   The area of each nucleus has been considered approximately as the area of the minimal rectangular envelope that would fit around it, as shown in Fig 2   The envelope has been located at a distance of 0 8 A exterior to the carbon skeletons (i e the contri-bution of the carbon atom to covalent bonds)   Hydrogen atoms have not been included in these calculations, as they contribute only about one-tenth as much as the heavier atoms to a van der Waals' adsorption
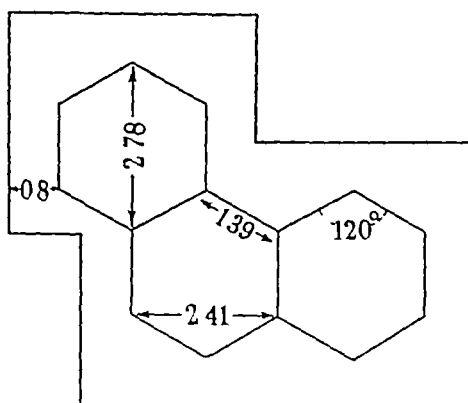
FIG 2 —Minimal rectangular envelope fitting a phenanthridine or angular benzquinoline nucleus
Dimensions in Angstrom units

TABLE VI —*Flat Areas of Molecules   Size of Minimal Rectangular Envelope which would contain Various Nuclei*

Substance	Dimensions in A	Area in Sq A
Pyridine	(3 97 × 4 38)	17 4
Quinoline	(6 38 × 4 38)	27 9
Acridine also 6 7 benzquinoline	(8 78 × 4 38)	38 5
Phenanthridine, also 5 6- and 7 8 benzquinolines		38 3
2 3 Benzacridine	(11 16 × 4 38)	48 9
1 2- and 3 4-Benzacridines		48 7
Flat surface of tetrahydroacridine		(as quinoline)
Flat surface of tetrahydrobenzacridine		(as acridine)
2-Styrylquinoline		49 9
2 6 Distyrylpyridine		61 6
Phenyl radical		(as pyridine)
Naphthyl-radical		(as quinoline)
Anthryl radical		(as acridine)

It is seen from Table VI that the critical size, above which a flat kation becomes antibacterial, lies somewhere between the 28 sq A of quinoline and the 38 sq A of acridine   The necessity for this minimal flat area is obviously to provide a sufficiently great area for adsorption on to a biological surface

Increasing the number of atoms in a molecule increases its chances of adsorption because the small van der Waals' forces, which unite any atom of the molecule to any atom of the bio-receptor surface, become collectively a really strong force when a large number of atoms are concerned on each side. This force is opposed by the kinetic energy of translation of the molecule, which does not increase as the size of the molecule increases. Hence there are critical sizes above which various molecules are likely to remain absorbed. The special requirement of the present series, viz. that the atoms responsible for this van der Waals' adsorption should all lie in one plane, strongly suggests that the atoms in the biological surface with which they have to make contact also lie in one plane.

The paramount requirement, that the molecule should be well ionized, proves that mere van der Waals' adsorption is not the biologically injurious act. As has been shown for the acridines in Part II, the biologically injurious act is a union (by salt-linkage) between the kationic portion of the drug (which for practical purposes may be considered as being, principally, the ring-nitrogen) and an anion which it is vitally important for the bacterium to have free. The function of the van der Waals' adsorption is to make more permanent the very transitory attachment effected by a salt-linkage in an ionic environment.

That there is no necessity for the kationic portion of the antibacterial substance to coincide with the area of van der Waals' adsorption is demonstrated by substances in which these two portions are at a distance from one another, e.g. anthrylguanidine (No. 72) anthryldiguanide (No. 75) and Ω-amino-5-ethylacridine (see Part II). However, activity of a really high order such as that shown by 5-aminoacridine and the highly-ionized benzacridines most likely depends not only upon the kationic portion falling within the area of van der Waals' adsorption, but even upon its occupying a particular part of that area.

Given these two essential requirements for antibacterial activity in heterocyclic bases, viz. adequate kationic ionization and an adequate area of flat surface, it is interesting to see what other heterocyclic families have these requirements. A survey has recently been made of the basic strengths of 23 other heterocyclic series (Albert *et al*, 1948), and it has been found that almost all of the examples (which included the most basic amino-derivatives of each series) were such weak bases that one of the above requirements could not be fulfilled. The principal base-weakening feature in a heterocyclic molecule was found to be the presence of two hetero-atoms in the one ring. The almost complete lack of basic properties in nitrogen, when situated in a 5-membered ring, further reduces the choice of series. No. 76–84, in Table V, give a sample of this class of material. Most of the examples chosen also lack the minimal area of flat surface, being as poorly off as quinoline in this respect. As there is usually little variation in pK_a among benzologues, there is no reason to suppose that the addition of another ring to these substances (No. 78 possibly excepted) would make them active antibacterials.

Up to this point we have been discussing a high degree of ionization introduced by the phenomenon of additional ionic resonance (Part II), but two other methods are sometimes practicable, (a) a ring-nitrogen can be made quaternary, or (b) a basic side-chain can be built on to the molecule. Although the quaternization of a nitrogen atom can be relied upon to provide 100 per cent ionization in the aliphatic series this is not a reliable device in heterocyclic series. Quaternary heterocyclic bases, when held at pH 7, usually undergo a chemical rearrangement

whereby an equilibrium is established between the original base and a derivative in which a hydroxyl-group has become covalently bound to a distant carbon atom, giving a " carbinol " As a consequence of this reaction an equilibrium pK_a is established, in some cases within a few seconds, in others after many hours This new pK_a may prove too low to be useful, but even when this objection does not apply a fresh difficulty often arises, namely the carbinol autoxidizes to the corresponding (non-basic) ketone and hence the material is irretrievably wasted Both of these difficulties were discussed in the case of methylacridinium salts in Part II Yet, in spite of them, one moderately active substance was found which owed its high basic strength entirely to quaternization (3-amino-5 10-dimethylacridinium bromide), and a few other cases are known where anti-bacterial activity can be attributed to a successful introduction of a high degree of ionization by quaternization Some moderately antibacterial quaternary styrylquinolines and quaternary phenazines have been described by Browning, Cohen, Ellingworth and Gulbransen, 1926, and Browning et al , 1922, respectively The non-quaternary analogues were inactive, which is not surprising, as they are now known to be poorly ionized

The second alternative, insertion of a basic side-chain, can also cause difficulties, viz (i) An Ω-aminomethyl-group is often unstable on a highly conjugated ring , however, an Ω-aminoethyl- or a guanidyl-group is stable , (ii) Ω-amino-alkyl-groups are susceptible to destruction by amineoxidase , however, it is not established that this enzyme can operate on heterocyclic examples , (iii) biologically-inactive micelles may be formed by hydrogen-bonding from the ionized nitrogen of the side-chain to the non-ionized negatively-charged ring-nitrogen , this effect was encountered (and demonstrated potentiometrically) in 3-acridyl-guanidine and 3-acridyl-N^1-diguanide but it did not occur in 5-Ω-aminoethyl-acridine (it has also been encountered, in the absence of a basic side-chain, but for the same reasons, in 1-amino-p-phenanthroline)

One further general difficulty must be mentioned, although it may not often be encountered outside the thionine and oxonine series These series have reduction potentials near to zero, so that they are easily reduced by a number of organisms Thionine and methylene blue (No 85 and 86) may be taken as examples Although these molecules have the right basic strength and the necessary flat area, their E_0 values at pH 7 are $+ 0 06$ and $+ 0 01$ volts respectively When reduced, their pK_a values are only 5 3 and 5 8 respectively (Phillips Clark and Cohen, 1927), so that they then cease to be ionized In the course of the bacteriological trials recorded in Table V, partial or complete reductions were recorded which explains the low antibacterial values found The acridine antibacterials have reduction potentials below $- 0 47$ volts (Part II), and those of the amino-benzquinolines, -acridines, -phenanthridines, -quinolines and -pyridines as well as various phenanthrolines and substances 76 to 83 in Table V fall well below this figure (J B Willis, private communication) Hence wastage of these substances through biological reduction is not to be expected

In spite of the various difficulties outlined above, it should not be thought that the families listed in Tables I, II and IV exhaust the possibilities for finding new types of highly-active kationic antibacterials in the heterocyclic series On the contrary, consideration of the above principles should help in the discovery of fresh examples

TABLE VII —*The Effect of Serum on the Antibacterial Action of Simple Aliphatic Amines Highest Dilutions Completely Preventing Visible Growth in 48 hours at 37° C (pH 7 2–7 4)*

Medium A Plain broth
B 10 per cent serum broth

No	Substance	Med ium	Cl tret chii	Str pyo- genes	Staph aur- eus	B coli	Pro teus	Bacteriostatic index (sum total of code numbers of inhibitory dilutions)	pKa in water at 20° C *	Per cent ionized (kation) at 20° C, pH 7 3
						Organisms				
87	Undecylamine	A	4	5	3	3	2	17	10 7	100
		B	3	1	0	0	0	4		
88	Dodecylamine	A	6	5	4	4	0	19	10 7	100
		B	1	0	0	0	0	1		
89	Hexadecylamine (Cetylamine)	A	3	4	3	0	0	10	10 7	100
		B	3	3	3	0	0	9		
90	Cetyltrimethyl- ammonium bromide	A	5	6	6	1	0	18	>14	100
		B	4	5	3	0	0	12		

* Hoerr, McCorkle and Ralston (1943)

(iii) Simple Aliphatic Amines

It is known that the antibacterial action of simple aliphatic amines increases as the pH of the medium is increased (Gershenfeld and Milamck, 1941) Acridines behave in the same way It was shown in Part II that this is because the increasing alkalinity, although leaving the ionization of the acridine unchanged, increases the ionization of the weakly acidic groups—(from the data of Part II, the pK$_a$ of these groups is above 9 They could be the — OH groups of tyrosine, purine or pyrimidine residues)—on the bacterial surface and hence facilitates the combination It is pertinent to inquire whether the antibacterial action of these aliphatic bases, some typical examples of which are shown in Table VII, has anything further in common with the flat heterocyclic bases which we have been considering

It has been shown that, as the chain length is increased, the aliphatic mono-amines become highly antibacterial above the C$_9$ homologue (Fuller, 1942) Activity tends to fall off with higher members (about C$_{16}$), a fact which is apparently due to micelle-formation Stereochemically, it should be noted that the aliphatic bases are not flat in any part, on the other hand, they are extremely flexible, whereas ring-systems possess a high degree of rigidity

No attempt has yet been made to obtain a series of aliphatic bases of varying pK$_a$ values and then to seek a connexion between ionization and antibacterial activity The effect of increasing alkalinity makes it fairly certain that such a connexion would be found However, an important difference between the aliphatic bases (Table VII) and the flat heterocyclic bases listed in Tables I to IV is that the former, when highly active antibacterially, are extremely susceptible to the presence of serum (cf in particular No 87 and 88, Table VII), but the latter are all unaffected by serum

It is evident that the flat heterocyclic bases have a special feature which makes them less ready to combine with serum proteins but no less ready to combine with the vital receptors on the bacteria This feature is, most likely, of a steric nature

A key is attached for interconversion of the British system of numbering heterocyclic rings, as used in this paper, and the American system (Table VIII)

13

TABLE VIII —*Key for Interconversion of the British System of Numbering (as Used in this Paper) and the American System*

British Name	American Name	1	2	3	4	5	6	7	8	9	10	1'	2	3	4
Acridine	Acridine	4	3	2	1	9	8	7	6	5	10				
5 6-Benzquinoline	Benzo[f]quinoline	4	3	2	1			6	5			10	9	8	7
6 7-Benzquinoline	Benzo[g]quinoline	1	2	3	4	5			10			6	7	8	9
7 8-Benzquinoline	Benzo[h]quinoline	1	2	3	4	5	6					7	8	9	10
Phenanthridine	Phenanthridine	4	3	2	1	10	9	8	7	6	5				
1 2-Benzacridine	Benz[c]acridine			5	6	7	8	9	10	11	12	1	2	3	4
2 3-Benzacridine	Benz[b]acridine	6			11	12	1	2	3	4	5	7	8	9	10
3 4-Benzacridine	Benz[a]acridine	6	5			12	11	10	9	8	7	4	3	2	1

(Left-hand vertical label: American numbering)

SUMMARY

1 Eighty heterocyclic bases having flat, rigid molecules have been examined for antibacterial activity (Tables I to V)

2 As with the acridines, antibacterial activity has been found to depend on a high degree of ionization at the pH of the tests (pH 7 3)   It is shown that at least 50 per cent ionization at 20° C (i e 33 per cent at 37° C) is necessary before the full antibacterial potentialities of each series are approached (see particularly Tables I and II)

3 It has been shown that the molecules of heterocyclic bases, even when highly ionized, must possess a minimal flat area (above 28 sq A) before powerful antibacterial properties appear (see Table IV)   The acridines the three series of benzquinolines, the phenanthridines and the three series of benzacridines all possess this requirement   Highly-ionized members of other series which fall below this requirement (quinolines, pyridines, aryl-guanidines and aryl-diguanides) are not normally antibacterial, but acquire activity when a sufficiently large co-planar area is built on to the molecule

4 Other factors which may influence the antibacterial activity of heterocyclic bases are discussed and some comparisons with the simple aliphatic bases are made

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ANTIBIOTICS ACTIVE AGAINST BACTERIAL VIRUSES *

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THE search for antibiotics against viruses requires new methods for detecting the presence of active substances in a reasonably short time, that is, before the product tested deteriorates Direct tests with human and animal viruses are not yet suitable, being too slow and cumbersome for a routine screening method On the other hand, bacterial viruses, i e bacteriophages, seem to be quite suitable for this purpose, provided that we make a totally unproved assumption that at least some of the animal and human viruses will be as sensitive to certain antibiotics as bacteriophage There are no ways to prove or disprove such an assumption, even by analogy, until active antibiotics are actually found and tried Furthermore, we are of the opinion that even if the antiphage substances discovered prove to be inactive against any of the other viruses, they will provide new interesting agents for the study of host-parasite relations as well as for the general study of cell biology At the same time, from the technical point of view, bacteriophage is an ideal test virus giving test results in less than 24 hours, and it can be as easily handled as ordinary bacteria

Therefore, our plan in search for antibiotics was to use bacteriophages as simulants of pathogenic viruses for screening purposes, for the follow-up of the production of such antibiotics, and for the basic studies of the mode of action of substances interfering with virus development

METHODS

Mould cultures were usually isolated from plate contaminations and were not identified before trials

Every mould tried was grown on four different media

1 Papain meat digest (Papain broth) (Asheshov, 1941), pH 7 0

2 Same with addition of 2 per cent sucrose

3 A modification of Czapek-Dox medium

Sodium nitrate	3 g
Monopotassium phosphate	1 g
Potassium chloride	0 5 g
Magnesium sulphate	0 5 g
Corn syrup	20 g
Water	1000 ml

Lactic acid, sufficient to bring the reaction to pH 4 5

4 Our own synthetic medium referred to as "No 1 medium "

Calcium lactate	1 g
Monopotassium phosphate	1 g
Ammonium nitrate	0 5 g
Magnesium sulphate	0 25 g
Corn syrup	20 g
Water	1000 ml

Lactic acid, sufficient to bring the reaction to pH 4 5

Media were distributed in 250 ml amounts into Roux bottles and autoclaved at 118° C for 20 minutes

Flasks were inoculated by what we call "foam method," which differed depending on the nature of the medium inoculated To papain broth a few ml of a heavy suspension of mould spores were added The bottles then were vigorously shaken until a thick layer of foam was produced The foam, containing spores, disintegrated very slowly and disappeared only after the spores began to germinate This fact ensured an even and dense film of surface growth Synthetic media were inoculated differently Since these media do not contain protein products a solution of peptone deposited on the surface of such media immediately spreads over the whole surface in a thin film Utilizing this fact, we prepared our spore suspension in either chilled broth or in peptone water By means of a pipette, foam was produced of which one or two pipettes full (about 3 ml in volume) were placed on the surface of the media, where it immediately spread over the whole surface Such bottles, left undisturbed, yielded again an even and dense film of surface growth

Samples of the culture media were collected as soon as the growth of the mould suggested that sufficient metabolites were present Sampling was repeated at regular intervals, i e two or three times a week

The samples were examined by the paper disc method on agar plates and, when necessary, by the serial dilution method in a special broth

Paper disc method

Plates for the paper disc method were prepared in the following way

Medium　Papain broth (Asheshov, 1941)　　　　　　　　　　1000 ml

　　　　　Bacto Yeast Extract　　　　　　　　　　　　　　　　2 5 g

　　　　　Agar (" K ' by Agar Products Co , Los Angeles or

　　　　　　Difco)　　　　　　　　　　　　　　　　　　　　　9 g

　　　　　pH 7 0

The medium was sterilized only once and then distributed into petri dishes, 30 ml per plate, using a Macalaster Bicknell 850 automatic pipette

Just before use the plates were flooded with a suspension of bacteria, to which bacteriophage was added in sufficient numbers to produce almost confluent clearings　Special paper discs were soaked in the liquid to be tested and deposited on the surface of the plates　Two kinds of paper discs were used　large discs, $\frac{1}{2}$ in in diameter, made by Schleicher & Schuell (740–E), and small ones, $\frac{1}{4}$ in in diameter, punched from filter-paper, S & S 598, with an ordinary office looseleaf punch　Small discs were used in testing new moulds, giving us an opportunity to examine 16 samples (four moulds on four different media) on one plate　The larger discs were used in testing samples already known to be active, thus providing a certain degree of quantitative estimation　Five to six discs could be used conveniently on one plate　After overnight incubation, provided the samples were active, the phage was suppressed and the discs were surrounded by an area of normal growth of bacteria, while the rest of the plate was covered by a layer of bacteria eaten out by the phage clearings (Fig 1, 2 and 3)

Serial dilution method

The following medium was used

　　　　　　Papain broth　　　　　　　　　1000 ml

　　　　　　Dextrose　　　　　　　　　　　10 g

　　　　　　NaCl　　　　　　　　　　　　　8 5 g

　　　　　　Brom Cresol Purple 0 1%　　　10 ml

　　　　　　pH 7 0

It was distributed into 16 × 150 mm tubes in 10 ml for the first tube of the series and in 5 ml portions for the rest of the series, and autoclaved

Usually a two-fold dilution series of antibiotic was prepared　Then approximately one million bacteria per ml and a number of phage corpuscles, sufficient to produce complete lysis in 18 hours, were added to each tube　This number was variable with different types and races of phage and with different strains of bacteria, and was determined experimentally before the test was run

The results of the dilution test usually showed zonal effect　Since culture fluids and even purified fractions could have, beside antiphage action, also antibacterial effect, tubes containing higher concentrations could be devoid of bacterial growth, showing clear purple medium　With increased dilution the antibacterial effect would be eliminated　Those tubes would be turbid and yellow—bacteria developing freely as bacteriophage was suppressed　On further dilution, antiphage action, in its turn, would be eliminated—phage lysing the bacteria　These last tubes would be again clear and purple
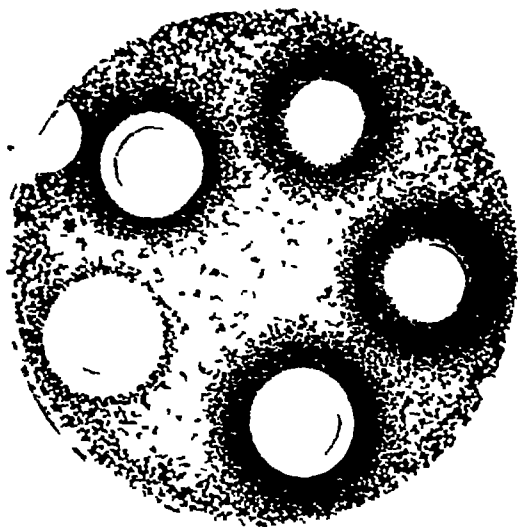
Fig 1
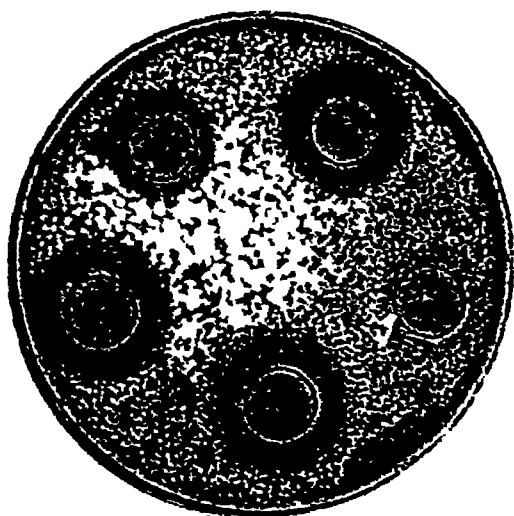
Fig 2
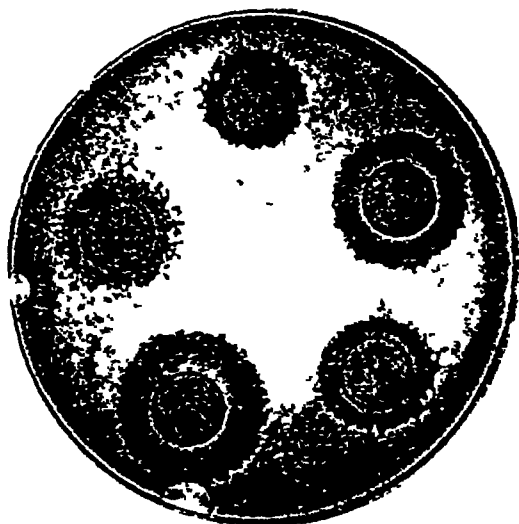
Fig 3

Fig 1 —Assay on staphylophage   Two of the discs show combined action on staphylococci and on staphylophage   One disc shows almost pure antistaphylococci action   Two discs show pure antistaphylophage action

Fig 2 —Assay on streptophage   Clearings of the phage are almost confluent

Fig 3 —Assay on streptophage   Phage destroying all bacteria outside the influence of antibiotic in the discs

The pH of the samples to be tested by disc or serial dilution methods was determined electrometrically   If the reaction fell below pH 6 0, it was brought up to about pH 7 0 by addition of a drop or two of a phosphate solution

Potassium monophosphate	.	10 g
Phenol red 0 05% sol	.	100 ml

It was distributed into small test-tubes in 2 ml portions and autoclaved   By adding phenol red, it was possible to see when approximate neutrality was reached

At the beginning of the work we used as test viruses only one staphylophage and three coliphages, occasionally including phages of the *Bacillus* group (*B cereus, B megatherium, B pumilis* and *B brevis*)   Later on, when the investigation showed considerable variations of sensitivity to antibiotics of different races and types of phages, we increased in our screening tests the number of different phages to 51   The following phages were used   23 types of staphylophages ,   6 types of streptophages ,   7 types of enterococcus phages ,   14 coliphages ,   1 type of typhiphage and 1 *B cereus* phage   Some of these phages were isolated by us   The majority were received from Dr N A Boulgakov, Laboratoire du Bactériophage, Paris, France, to whom we express our sincere gratitude as well as our admiration for saving and maintaining such a valuable collection of bacteriophages through the entire war, German occupation, and the difficult post-war period   We are indebted to Dr A Schatz for three phage cultures, and to Dr M Delbruck for the T series of the *Bact coli* phages

By employing these methods we examined only 30 moulds in all   Early in the investigation we found a mould which produced very active antiphage substances and our search for new antibiotics became sporadic, time being consumed by the study of the newly-discovered substances   Only four other moulds showed some doubtful activity against some of the phages, but these have not yet been investigated any further

The mould which showed considerable activity was an *Aspergillus* sp   It produced at least five different antibiotic substances at different stages of its development   Three of those substances were active only on bacteria, one active against some streptophages (to a lesser degree also against coliphages T2, T4 and T6), and one was active against several staphylophages

THE PRODUCTION AND THE PROPERTIES OF ANTIPHAGE SUBSTANCES

The antibiotic, active against several races of streptophages, is produced early in the development of the mould   Its activity can be demonstrated generally on the third or fourth day of growth   It reaches its peak between the 6th and 10th day, when the pH of the medium has dropped to its lowest level (approximately pH 3 5)

The active substance is extracted with chloroform from the culture fluid at alkaline reaction (pH approx 10)   The extract is concentrated, washed with dilute hydrochloric acid and treated with bentonite, which removes some inactive coloured substances   The chloroform extract is dried and the residue is dissolved in benzene   The benzene solution is extracted several times with dilute ammonia, and is subsequently treated with norit until it is colourless

After the evaporation of the solvent, a gummy, pale yellow residue is left

which is almost insoluble in water, soluble in acetone, ether and most organic solvents except petrol ether

It inhibits the development of streptophage in dilutions 1 in 10 to 1 in 20 million by weight   This represents a 1000- to 2000-fold purification of the starting material   The yield in most cases is close to 100 per cent

By repeating the norit treatment of a more concentrated solution, using larger amounts of material, the activity can be increased to 1 in 40 million

The substance is stable between pH 2 and 6   A slow decline in activity takes place at neutral and weakly alkaline reaction, while strong alkali (above pH 9) destroys it almost immediately

Attempts to obtain the substance in crystalline form so far have been unsuccessful

The second antibiotic, active against several strains of staphylophage, is produced during the third week of incubation   It is generally harvested between the 18th and 22nd day when the pH of the medium has returned to neutral The culture fluid is acidified to pH 2 and the active substance is precipitated with phosphotungstic acid   The precipitate is dissolved in dilute ammonia, acidified and extracted with carbon tetrachloride   The active substance is extracted from this solvent with dilute ammonia   The aqueous solution is again acidified and extracted with benzene   The solvent is evaporated, the residue dissolved in dilute ammonia, acidified and extracted with ether   On evaporation of the solvent the active substance crystallizes in colourless, blunt-edged needles   They are washed with cold alcohol and recrystallized from hot 95 per cent alcohol   The crystalline material inhibits the development of staphylophages in dilution 1 in 20 million by weight, while approximately a hundred times higher concentration is required for a bacteriostatic effect on staphylococci   It does not show any action on streptophage or *Bact coli* phage

The substance is sparingly soluble in water, easily soluble in dilute alkali, and most organic solvents except petrol ether   It is fairly stable in aqueous solution between pH 4 and 9, but is slowly destroyed by strong acid and alkali Heating to 100° C for 15 minutes does not destroy its activity   It reduces permanganate in the cold and gives a yellow precipitate with $FeCl_3$ It is optically inactive   It has no sharp melting-point, but decomposes at approximately 160° C

A more complete chemical characterization will have to await the accumulation of more material

As soon as we were in possession of antibiotics against the two types of phages, i e an anti-staphylophage agent which was found first and an anti-streptophage agent found one year later, we began our investigation to determine the nature of the antiphage action   It was realized that the suppression of the bacterial virus could be the result of action on the virus itself or on the host cell (bacterium), or on both   It could be possible that the antibiotic, without noticeably affecting the development of bacteria, affects their metabolism in such a way as to render them unsuitable as hosts for the virus   for instance, by blocking some enzyme system required by the virus   On the other hand, the antibiotic may influence the development process of the virus itself   It may also affect the liberation of the grown phage from bacteria by interfering with the lytic action of bacteriophage

In an effort to elucidate these problems we began a series of experiments

presented in this paper, which do not yet solve the problem, but throw some light on the nature of the action of the antibiotic on the host-parasite system The experiments were carried out with both antibiotics and it is noteworthy that both showed a remarkable similarity in their action in spite of their entirely different chemical nature

1 Is the bacteriophage corpuscle attacked by our antibiotics?—To a phage suspension, free from bacteria and containing about 1×10^8 phage particles, 50,000 units of antibiotic was added and was left to stand overnight at 37° C If the highest active dilution of a liquid or of a dry fraction was, say, 1 500, we referred to it as containing 500 units An identical suspension without antibiotic was used as a control On the following day the phage particles present were recounted, any antibiotic action being eliminated by the dilution used On no occasion was any decrease in the number of phage corpuscles noticed We concluded that our antibiotics do not attack phage corpuscles, at least when they are in a resting or " dormant " state

2 Does the antibiotic render the bacterial cell permanently unsuitable for phage development?—To determine this, bacteria were grown in the highest concentration of antiphage substance visibly not interfering with the normal development of bacteria After 24 hours the bacteria were washed, resuspended in broth and inoculated with phage The development of phage proceeded normally on the bacteria This experiment indicates that the antibiotic does not induce hereditary damage to the bacterial cell

3 Do our antibiotics interfere with adsorption of virus particles on bacteria? —Two identical suspensions of sensitive bacteria were prepared Antibiotic was added to one of the suspensions in an amount not sufficient to influence the vitality of the bacteria, but exerting definite antiphage action—usually about 10 units Both were then inoculated with phage After several minutes, depending on the known adsorption time of the particular phage, both suspensions were centrifuged, the supernatants separated, and the sedimented bacteria resuspended in the same amount of broth Supernatants and resuspended bacteria were plated for counting Several different strains of staphylophage and streptophage, with their corresponding strains of bacteria, were treated in this way On no occasion did we notice any interference with adsorption

Examples

Exp 1 Staphylococci plus staphylophage plus 10 units of antibiotic After 10 minutes 86 per cent of phage corpuscles were adsorbed on the bacteria in the presence of the antibiotic and 78 per cent in the control tube

Exp 2 Another strain of staphylophage with a different strain of staphylococci gave 73 per cent adsorption in the presence of the antibiotic and 80 per cent in the control tube

Exp 3 Enterococci phage 10 units of antibiotic After 10 minutes 100 per cent adsorption took place in the presence of the antibiotic and 95 per cent in the control tube

Exp 4 Coliphage T2, 2 units of antibiotic (the higher concentration had antibacterial action) After 10 minutes 100 per cent adsorption took place in the presence of the antibiotic and 97 per cent in the control tube

It may be concluded from these experiments that our antibiotics, in concentrations several times stronger than those necessary to suppress the development

of phage, do not interfere with adsorption of the virus particles on bacteria, and therefore, in all probability, do not appreciably alter the surface of the bacterial cell
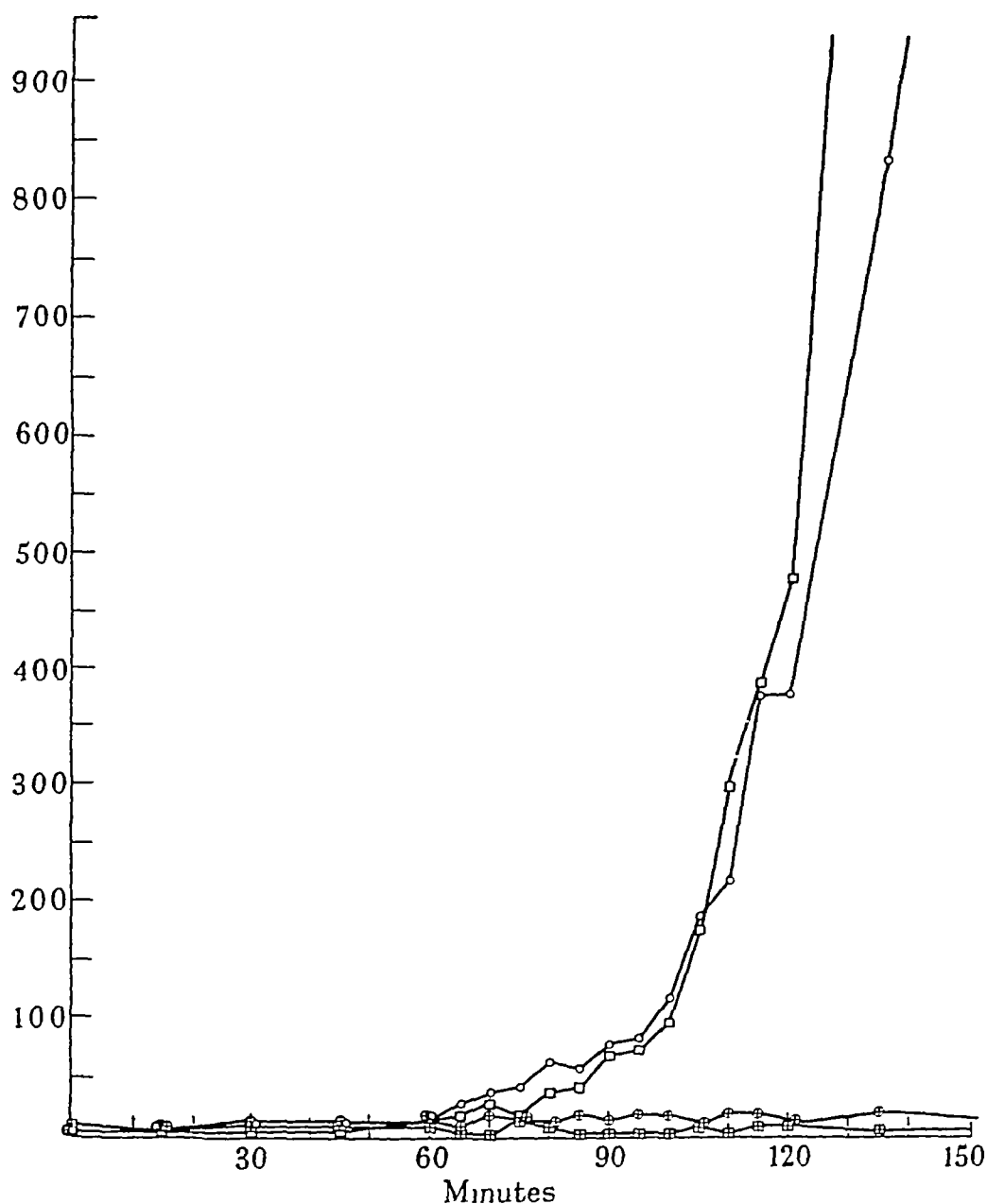
Fig 4 —" Sensitive " staphylophage εφ18 developing on two different strains of staphylococci (ε4 and ε18) in presence and in absence of antiphage substance (αφ)

Tube 1	φ18/ε4	□
Tube 2	φ18/ε4 + αφ	☒
Tube 3	φ18/ε18	○
Tube 4	φ18/ε18 + αφ	⊕

4 Is the sensitivity of different races of phage influenced by the use of different strains of host cells?—We found an exceedingly variable sensitivity to our antibiotics among the 20 or more different staphylophages investigated

Some phages were as sensitive as our test phage, some were almost completely resistant   As every staphylophage was propagated on its corresponding staphylococcus strain, we were able to decide, by following the development of a sensitive phage in the presence of antibiotic on the strain of staphylococci on which another phage proved to be resistant, whether the different sensitivity depended on the virus itself or on the host cell   By reversing the experiment we followed
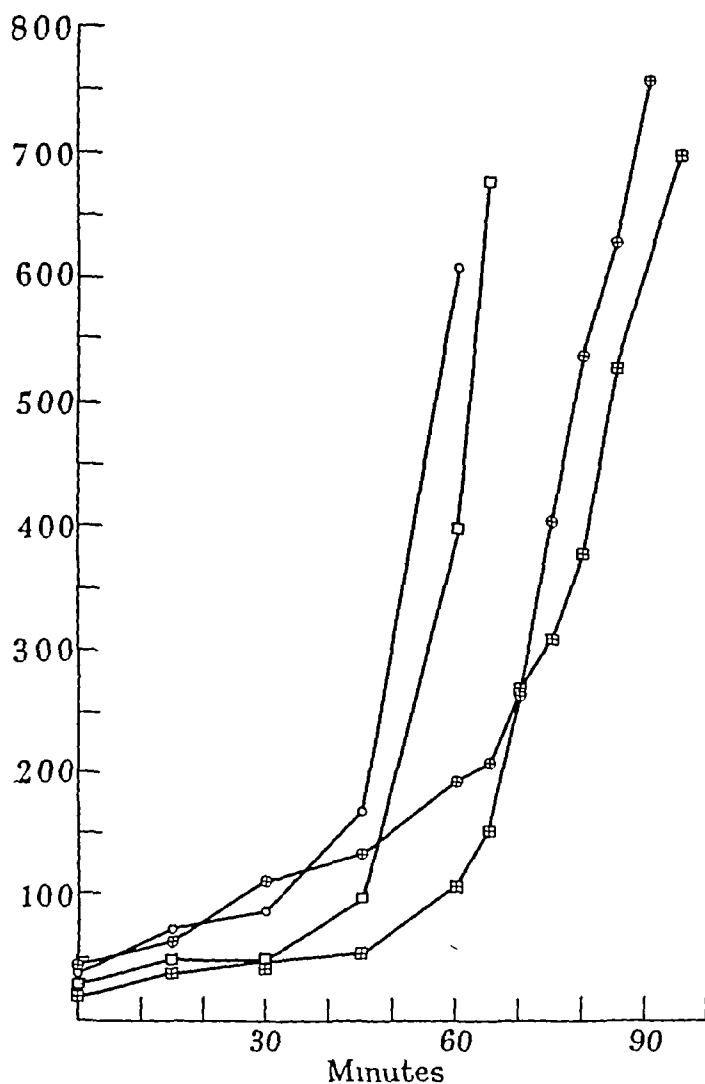
FIG 5 —" Resistant " staphylophage εφ13 developing on the same two strains of staphylo
cocci (ε4 and ε18) in Fig 4 in presence and in absence of antiphage substance (αφ)

Tube 1	φ13/ε4	□
Tube 2	φ13/ε4 + αφ	⊠
Tube 3	φ13/ε18	○
Tube 4	φ13/ε18 + αφ	⊕

the development in the presence of the antibiotic of a resistant phage on a bacterial strain on which another phage was suppressed   Several combinations were tried   Fig 4 and 5 illustrate a typical experiment   In Fig 4 a sensitive staphylophage Σφ18 shows normal development on two strains of staphylococci, Σ18 and Σ4, but there is no development on either of the strains of staphylococci

in the presence of 2 5 units of the antibiotic   When another staphylophage, $\Sigma\varphi13$, was tried on the same two cultures (Fig 5), its development was only slightly retarded by 2 5 units of the antibiotic in both cases   In other words, the resistance or sensitivity of a phage is maintained regardless of the strain of bacteria used to propagate it

These experiments suggest, but do not prove definitely, that the action of the antibiotic is directed towards virus corpuscles and not towards the bacterial cell   The two phages, $\varphi\Sigma18$ (" sensitive ") and $\Sigma\varphi13$ (" resistant ") may require a different activity of the bacterial cell for their development, and our antibiotic may be suppressing only the one required by $\Sigma\varphi18$, not affecting the cell process required by the " resistant " $\Sigma\varphi13$

5 Is the activity of the antibiotic against phage influenced by the number of bacteria used?—Several identical serial dilution sets of antibiotic were prepared, equal number of phage corpuscles were added to all sets, but a different amount of bacteria was used in each set   The end-point of the antiphage activity was the same in all sets

6 Is the activity of the antibiotic influenced by the number of bacteriophage corpuscles used?—The previous experiment was repeated but an equal number of bacteria were used throughout the sets, the number of phage corpuscles being varied in each set   It was found that an increase in the number of corpuscles from 3×10^4 to 3×10^5 brought about a 50 per cent decrease in the activity of antibiotic, while a hundred-fold increase in the number of phage particles from 3×10^4 to 3×10^6 caused a 90 per cent decrease in the antibiotic activity

7 Experiments were carried out to determine the influence of the addition of antiphage substance at different periods of phage development, but, before the first burst took place   A phage with a pre-bursting time of approximately 55 minutes was used   Five tubes with bacterial emulsion in broth were inoculated with bacteriophage   To one the antibiotic was added immediately after inoculation, the second received it after 15 minutes, the third after 30 minutes and the fourth after 45 minutes, i e 10 minutes before the bursts were expected to start   The fifth, or control tube, received no antibiotic   Only a few of the experiments carried out seemed to indicate that antiphage substance added at the time of infection, and 15 minutes after infection, prevented the bursts from taking place   Antibiotic added 30 minutes after infection probably allowed a few bursts to take place , there was a slight increase of the phage after one hour but no further development occurred   Antibiotic added 45 minutes after infection did not prevent the first burst, but after that there was no further development   This experiment is of particular interest as it shows that, in all probability our antibiotic interferes with the actual development of the phage either directly or indirectly and not with its liberation from bacteria

8 Experiments also were conducted with the view to learn what would happen if a bacteria-virus complex submitted to the influence of antibiotic is later freed from the antiphage agent   All these experiments, performed with the crude metabolites (culture liquid) and not the purified substance, were not always consistent probably due to the influence of other antibiotics present However the general tendency observed was that the phage, after having been freed from antibiotic resumed its development, but only after a lag period   This lag period was never shorter than the pre-bursting time specified for each phage but often longer   In some instances phage did not recover but, as

mentioned, this may have been due to the presence of impurities   These experiments again seem to support the point of view that the antiphage substance interferes with the actual process of growth, 1 e multiplication or formation of phage corpuscles inside bacteria

Since the majority of the experiments described in this paper were carried out with either crude culture media or with only partly purified concentrates, they all are only of a preliminary nature and are to be repeated using purer substances when they will be available   At the same time it must be pointed out that the complexity of the phenomenon of the antiphage action is considerably greater than it seems at first glance   We have to deal with, observe, and study the living progress of two interdependent and continuously variable subjects—living bacteria and their living parasite, bacteriophage   We have learned only the elementary facts in regard to their relationships, in spite of some 30 years of study   Every step in our investigation requires additional study of the basic phenomena of the activity of bacterial viruses and, therefore, our progress is of necessity very slow   At the same time, our impression is that all these experiments are contributing to the better understanding of the biology of the host-parasite relationship in general

SUMMARY

Two different antibiotic substances active against some bacterial viruses were found to be produced by an *Aspergillus* sp   The procedure of their isolation and some observations on their action on bacteriophages are described

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THE ERYTHROCYTE RECEPTOR FOR PERTUSSIS HAEMAGGLUTININ

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IT has already been reported (Fisher, 1948) that saline suspensions of the acetone-soluble lipids of human erythrocyte stroma inhibit, in high dilutions, the action of pertussis haemagglutinin, and that the same suspensions act as lipid haemagglutinins, 1 e they agglutinate the erythrocytes of certain fowls (Burnet and Stone, 1946)   This paper deals with further work aimed at identification of the active principles, which will be referred to as " inhibitor " and "agglutinin "

MATERIAL AND METHODS

Acetone soluble erythrocyte lipids

Mixed, unwashed, lyophilized human erythrocytes were extracted with boiling acetone, using either a continuous process, or three successive lots of solvent

The acetone soluble lipids were recovered by evaporation of the solvent on the water bath

Pertussis haemagglutinin

Virulent pertussis organisms were extracted with M/1 NaCl (Keogh, 1948, unpublished), the organisms removed by centrifuging, and the clear supernates adjusted to contain 8 minimal haemagglutinating doses per drop   In some experiments the supernates of centrifuged fluid cultures were used as the source of haemagglutinin

Inhibitor and agglutinin

Lipoid suspensions were prepared, and inhibitor and agglutinin titrations set up, as described in the previous paper (Fisher, 1948)   Agglutinin titres are expressed as the reciprocal of the dilution, and inhibitor titres as the reciprocal of the dilution multiplied by the number of minimal doses of haemagglutinin used

RESULTS

The acetone extracts of erythrocytes yielded on evaporation a brown oily fluid from which a large amount of white solids separated on standing   Nitrogen could not be detected by the sodium fusion test   The phosphorus content was 0 5 per cent, corresponding to 12 5 per cent of phospholipid   (Phospholipids are somewhat soluble in hot acetone , Bloor, 1943)   The acetone-soluble material could be separated by extraction with aqueous methanol into fractions which were active and inactive in respect of inhibition and agglutination   The portion soluble in 85 per cent and that insoluble in 95 per cent methanol were both inactive, whereas the material insoluble in 85 per cent but soluble in 95 per cent methanol was highly active   The two inactive fractions, when re-combined, exhibited the high activity of the original acetone extract

In one experiment 400 mg of acetone soluble material was extracted with two 15 ml lots of 85 per cent aqueous methanol   The insoluble material was removed by centrifugation, and on evaporation of the solvent on the water bath, 176 mg of amorphous greenish-brown material was recovered (Fraction 1)   The insoluble residue was extracted with two 15 ml lots of 95 per cent aqueous methanol, and by evaporation of the solvent, after removal of insoluble material by centrifugation, 54 mg of yellowish mixed amorphous and crystalline material was obtained (Fraction 2)   The residue of almost white, mostly crystalline material weighed 175 mg (Fraction 3)

The results of quantitative Liebermann-Burchard tests on each fraction expressed as g cholesterol/100 g were   Fraction 1, 12 per cent , Fraction 2, 53 per cent , and Fraction 3, 78 per cent

Approximate estimations of unsaponifiable material were done on Fractions 1 and 3 by the following method   Samples (20–30 mg) were boiled in 15 ml of aqueous 1 per cent KOH for 5 minutes in a nitrogen atmosphere   The samples were cooled and extracted several times with diethyl ether and petroleum ether   The extracts were combined, the solvents evaporated, and the residue weighed   Fraction 1 contained 10 per cent and Fraction 3 90 per cent of unsaponifiable material

Saline suspensions of each fraction and of combinations of them were prepared

from their solutions in diethyl ether and tested for inhibitory and agglutinating activity   The results are shown in Table I

TABLE I —*Aqueous Methanol Fractionation of Acetone-Soluble Lipids of Erythrocytes*

Fraction	Solubility in aqueous methanol	Inhibitor titre	Agglutinin titre
1	Soluble in 85%	$<10^4$	$<10^4$
2	Soluble in 95%, insoluble in 85%	$1\ 6 \times 10^6$	$1\ 6 \times 10^5$
3	Insoluble in 85% and 95%	10^5	$<10^4$
Mixture of equal parts of Fractions 1 and 3		$3\ 2 \times 10^6$	$1\ 3 \times 10^6$

The bulk of the saponifiable material, most of the pigment and all the phosphorus were contained in Fraction 1, soluble in 85 per cent methanol   This fraction suspended well in saline, but was inactive as agglutinin or inhibitor

Fraction 3, insoluble in 95 per cent methanol, consisted mainly of unsaponifiable material, chiefly steroid   It gave poor saline suspensions, which were only weakly active

A mixture of these inactive fractions 1 and 3 gave good saline suspensions of high activity

Fraction 2, which physically and chemically was intermediate in composition between Fractions 1 and 3, gave well-dispersed saline suspensions of high activity

These results indicated that the inhibitory and agglutinating activities of the acetone-soluble portion of erythrocyte stroma were properties of a sterol, only slightly soluble in aqueous methanol, which required to be associated with saponifiable lipoids, relatively soluble in aqueous methanol, for exhibition of its full activity

In view of these findings, combinations of cholesterol with lecithin, and with oleic acid, were examined   Saline suspensions were prepared from diethyl ether solutions and tested for inhibitory and agglutinating activity   The results are shown in Table II

TABLE II —*Agglutinin and Inhibitor Titres of Suspensions of Cholesterol Containing Lecithin or Oleic Acid in Varying Proportions*

(a) *The lecithin-cholesterol series*

Reciprocal of (concentration $\times 10^4$)		Quality of dispersion	Agglutinin titre	Inhibitor titre
Cholesterol	Lecithin			
—	1	Good	—	—
16	1	,,	$<1\ 6 \times 10^5$	$1\ 0 \times 10^6$
8	1	,,	$<0\ 8 \times$,,	$2\ 0 \times$,,
4	1	,,	$<0\ 4 \times$,,	$6\ 4 \times$,,
2	1	,,	$12\ 8 \times$,,	$12\ 8 \times$,,
1	1	,,	$12\ 8 \times$,,	$12\ 8 \times$,,
1	2	,,	$6\ 4 \times$,,	$6\ 4 \times$,,
1	4	,,	$3\ 2 \times$,,	$3\ 0 \times$,,
1	8	Fair	$2\ 4 \times$,,	$1\ 0 \times$,,
1	16	,,	$1\ 0 \times$,,	$0\ 5 \times$,,
1	—	Poor	$0\ 05 \times$,,	$0\ 25 \times$,,

TABLE II—*continued*

(b) *The oleic acid-cholesterol series*

Reciprocal of (concentration × 10⁴)		Quality of dispersion	Agglutinin titre	Inhibitor titre
Cholesterol	Oleic acid			
—	1	Good	$(1 \times 10^5)^*$	$(0\ 1 \times 10^6)^*$
128	1	,,	$150 \times$,,	$6\ 0 \times$,,
64	1	,,	$70 \times$,	$6\ 0 \times$,,
32	1	,,	$60 \times$,,	$6\ 0 \times$,,
16	1	,,	$20 \times$,,	$6\ 0 \times$,,
8	1	,,	$20 \times$,,	$12\ 0 \times$,,
4	1	,,	$20 \times$,,	$7\ 7 \times$,,
2	1	,,	$10 \times$,,	$4\ 8 \times$,,
1	1	,,	$5 \times$,,	$3\ 6 \times$,,
1	2	,,	$6 \times$,,	$3\ 0 \times$,,
1	4	Fair	$20 \times$,,	$1\ 8 \times$,,
1	8	,,	$5 \times$,,	$0\ 9 \times$,,
1	—	Poor	$0\ 08 \times$,,	$0\ 05 \times$,,

* Refers to titre in terms of oleic acid concentration   Titres are based on cholesterol concentrations, except in the cases noted

In the lecithin-cholesterol series the agglutinin and inhibitory activities showed a maximum in the same region, where the components were in equal concentration, or where lecithin was in slight excess   In the oleic acid-cholesterol series conditions were more complex, since oleic acid itself has haemolytic and haemagglutinating properties   In both series the inhibitory activity was slight in the region of excess cholesterol

Palmitic acid and cephalin tested under the same conditions as oleic acid and lecithin failed to enhance the slight inhibitory action of saline suspensions of cholesterol

Several sterols and steroid derivatives were tested to determine which part of the sterol molecule is active in inhibition   The substances were dispersed in saline from their solutions in diethyl ether or ethanol, in combination with oleic acid or lecithin   Cholic and desoxycholic acids were dissolved in saline with the aid of a little alkali   The results are shown in Table III

TABLE III —*Inhibitory Power of Some Steroids against Pertussis Haemagglutinin*

Compound	Inhibitory titre
Cholesterol	10^7
Ergosterol	3×10^6
Calciferol	3×10^6
Corn germ sterol	6×10^6
Androsterone	$<10^5$
Cholesterol palmitate	$<10^5$
Cholesterol oleate	$<10^5$
Cholic acid	$\vee 10^5$
Desoxycholic acid	10^5
7-keto-cholesterol acetate	10^5

The substances which were active inhibitors, cholesterol, ergosterol, calciferol and corn germ sterol (presumably mainly stigmasterol), differ from the inactive substances in having three structural properties in common—(1) free hydroxy-group at C3, (2) double bond between C5 and C6, (3) presence of a side-chain

The same substances, except cholesterol oleate, were tested for their ability to inhibit the haemolytic action of digitonin, by a technique similar to that of Ponder (1945) Those substances which inhibited pertussis haemagglutinin also inhibited haemolysis by digitonin, and those inactive against digitonin failed to prevent haemolysis

DISCUSSION

The above results indicate that cholesterol is the inhibitor of pertussis haemagglutinin present in acetone extracts of erythrocytes The total amount of inhibitor which can be extracted from erythrocytes corresponds approximately to their cholesterol content, and cholesterol in association with lecithin or oleic acid inhibits to the same degree as the acetone soluble lipids The evidence that the inhibitor present in acetone extracts of erythrocytes is also the receptor on the cell surface was discussed in the previous paper (Fisher, 1948) The work of Stone (1946) shows that the lipid haemagglutinin is of similar nature, and our results are in conformity with her findings

Previous failure to recognize cholesterol as the inhibitor (Fisher, 1948) was due to two circumstances First the accessory but essential role of associated lipids in the preparation of cholesterol suspensions of maximum inhibitory titres had not been elucidated Secondly, it was not appreciated that digitonin may fail to precipitate cholesterol quantitatively from solutions of the acetone soluble lipids of erythrocytes Using the method of Kelsey (1939), it has since been observed that the percentage removal of inhibitor from such material may vary from nil to 90 per cent

Ponder (1945) has shown that the active patch for digitonin fixation in the steroid molecule is the C3-C5-C6 region The, evidence presented suggests that the same region is concerned in fixation of pertussis haemagglutinin, but the possibility has not been excluded that the nature of the side-chain may have a modifying action

SUMMARY

1 The inhibition of the haemagglutinin of *H pertussis* by the acetone-soluble lipids of human erythrocytes appears to be due to the cholesterol contained in the lipids

2 Cholesterol in saline suspension does not exhibit its full potential inhibitory action against pertussis haemagglutinin in the absence of the saponifiable portion of the acetone-soluble lipoids, for which lecithin or oleic acid may be substituted

I am indebted to Dr P Fantl for the gift of several samples of sterols and sterol derivatives

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14

ENZYME ADAPTATION IN BACTERIA
FATE OF NITRATASE
IN NITRATE-ADAPTED CELLS GROWN IN THE
ABSENCE OF SUBSTRATE

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SOME progress has been made in the study of enzymatic adaptation in micro-organisms by following the increase in specific enzyme activity which may occur on incubation or growth of cells in the presence of substrate   The reverse process of " deadaptation," however, has not yet been properly investigated   Although it is known that in many cases a single subculture of previously-adapted cells in a medium not containing the substrate is sufficient to cause loss of adaptation, little systematic work has been reported upon the nature or progress of this reversion

The autocatalytic nature of the curve of most enzymatic adaptations in washed suspensions of cells has led Spiegelman (1946) to suggest that an adaptive enzyme or its precursor might act under some circumstances as a self-reproducing entity   On the other hand, Spiegelman and Dunn (1947) have brought forward evidence supporting the concept of enzyme " interaction "—viz formation of a new adaptive enzyme at the expense of previously-formed enzyme when the substrate responsible for the first adaptation is replaced by a second substrate

The behaviour of an adaptive enzyme in adapted cells growing and multiplying in a medium without the substrate known to be necessary for maximal enzyme production must presumably correspond to one of the following three hypotheses

(1) The enzyme activity per cell may remain constant, or fall off slowly, such that there is an increase in the total amount of enzyme in the culture

(2) The preformed enzyme may be destroyed or inactivated so that the total enzyme in the culture diminishes

(3) The total enzyme may remain constant so that, since the cells are growing, the enzyme activity *per cell* will gradually decrease, and the deadaptation process will eventually be achieved by a simple " dilution out " of the adapted enzyme

If (1) were true, it would suggest that the enzyme or enzyme precursor, induced by substrate might be endowed with some independent self-reproducing properties   and would in any case be evidence in support of the direct effect of environment upon heredity   Hypothesis (2) is what might be expected in cases where adaptive enzyme interaction (Spiegelman and Dunn, 1947) occurred   Hypothesis (3) is what would occur if preformed enzyme remained

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unmodified—being neither inactivated nor " growing " with the cells—during increase of the rest of the bacterial protoplasm

The adaptation of the nitratase enzyme system has been studied in the presence of nitrate in non-proliferating washed suspensions (Pollock, 1946) and during growth (Pollock and Wainwright, 1948) using the Intermediate coliform bacterium " 1433 " The same cells can be adapted simultaneously and independently to tetrathionate reduction (tetrathionase) , and tetrathionate acts equally well as nitrate as H-acceptor for anaerobic growth with lactate or glucose It was, therefore, decided to study the problem of deadaptation by following the nitratase of nitrate-adapted cells inoculated into a medium containing tetrathionate that is, during growth in, and further adaptation to, a new substrate The problem was slightly complicated by the fact that there is always a certain small amount of nitratase activity developed, even though the cells are grown without substrate This, in practice, means that the total nitratase of a growing culture is always increasing, even in the absence of nitrate However, allowance can be made for this " basal " nitratase, and the results here reported show that the behaviour of adaptively formed nitratase corresponds closely with that which would be expected were the loss of adaptation to proceed by a process of " dilution out " of the preformed enzyme (Hypothesis 3)

METHODS

Organism —" 1433 "—a lactose-fermenting " Intermediate, Type I " strain of *Bact coli* (Pollock, 1946)

Suspensions —Prepared as previously described (Pollock and Wainwright, 1948) from the 16-hour growth at 35° on tryptic meat agar

Adapted suspensions were prepared by incubation for 3–3½ hours at 35° in $M/100$ glucose, $M/50$ sodium nitrate (or tetrathionate), $M/5$ phosphate buffer, pH 7 2 and $M/600$ MgSO$_4$, at a cell strength of 1 mg /ml The cells were then twice washed with distilled water Control unadapted suspensions had similar treatment, but without nitrate or tetrathionate

Nitratase activity was followed at 35° in evacuated Thunberg tubes with $M/100$ sodium nitrate and $M/50$ formate, as H-donor, in the presence of $M/800$ FeSO$_4$ Samples were removed at 30 min and 60 min and the nitrite was estimated by means of the Griess Ilosvay Reagent (Pollock, 1946) Results are expressed as μmol nitrite formed/mg dry weight of cells/hour Cell strength was determined by opacity measurements on the washed cell samples

Tetrathionase activity was estimated by iodine titration of thiosulphate formed during incubation of cells anaerobically, at a concentration of 1 mg /ml in Thunberg tubes, at 35° with $M/100$ sodium tetrathionate and $M/50$ formate, as H-donor (Pollock and Knox, 1943) Results are expressed as μmol thiosulphate formed/mg dry weight of cells/hour " *Total* " *nitratase or tetrathionase activities* simply refer to the total amount (in μmol) of nitrite or thiosulphate formed by the whole bacterial culture per hour

Growth medium for deadaptation experiments was as follows

$M/5$ phosphate buffer, pH 7 2, $M/50$ glucose, $M/50$ H-acceptor (sodium tetra-thionate or sodium fumarate), $M/100$ NH$_4$Cl and $M/600$ MgSO$_4$ Growth was followed anaerobically at 35° in 6 × ¾ in tubes, filled with argon (as described by Pollock and Wainwright, 1948) Up to 8 similarly prepared and inoculated

tubes were used for each curve, one tube being used for each sample   Initial concentration of cells was 50 μg /ml

Samples for nitratase determinations —1 ml of M/200 oxine (8-hydroxy-quinoline) was added to a whole tube of 10 ml to stop growth and further adaptation   The cells were centrifuged, washed once with water, made up to about 1 0 ml and the concentration estimated turbidimetrically

Growth was followed in the tubes by direct estimation of opacity on the " Spekker " absorptiometer by means of a specially-constructed tube adaptor (Pollock and Wainwright, 1948)   A standard curve of the relation between dry weight and opacity was prepared, and all results are given in terms of dry bacterial weight

<div align="center">RESULTS</div>

Absence of enzyme " interaction " in washed suspensions

As mentioned above, previous experiments (Pollock, 1946) had suggested that it was possible to further adapt nitrate-adapted cells of " 1433 " to tetra-thionate without significant loss of the original nitratase, and *vice versa*   In this respect, behaviour is quite different from washed suspensions of *Saccharomyces cerevisiae* adapted to galactose which lose galactozymase activity on being adapted, under similar conditions, to maltose (Spiegelman and Dunn, 1947) Table I gives the results of a more extensive confirmatory experiment, and shows

TABLE I —*Independence of Adaptations to Nitrate and Tetrathionate by Washed Suspensions of " 1433 "*

Cells further treated anaërobically for 3 hr at 35° at a conc of 1 mg /ml with

Cells previously adapted to	M/5 phosphate buffer (pH 7 2) M/600 MgSO₄ M/10 glucose	M/10 sod nitrate	M/10 sod tetrathionate	Nitratase activity	Tetrathionase activity
Tetrathionate	+	+	—	3 9	8 4
	+	—	—	0 2	7 4
	No further treatment			0 2	8 6
Nitrate	+	—	+	3 8	6 5
	+	—	—	3 8	0 3
	No further treatment			4 1	0 2

that there is no evidence of " interaction " between tetrathionase and nitratase in washed suspensions   The final enzyme activities attained with respect to both nitratase and tetrathionase differ little whichever substrate was added first   The further treatment of nitrate-adapted cells to tetrathionate does not significantly diminish the original nitratase activity, and *vice versa*   Moreover, previously adapted cells appear to adapt as well to their second substrate as do unadapted cells to their first   Both enzyme adaptations appear to proceed independently

The next stage was thus clearly to determine if such independence persisted during growth and cell multiplication   Here, however, it was only possible to follow the nitratase activities because the iodometric determination of thiosulphate is not sensitive enough to enable the tetrathionase activities of low concentrations of cells to be estimated accurately

Experiments with growing cells

Most of the experiments were done with tetrathionate as H-acceptor for reasons given above, but, in order to determine whether cells behave similarly in other media, in one experiment the tetrathionate was replaced by M/50 fumarate

Two difficulties were encountered from the beginning

(1) The problem of measuring nitratase activities of samples of cells at a particular point during growth

(2) A "dilution effect" Both dilution of cell suspensions below a concentration of 0 2 mg /ml and the centrifugation of cells from large volumes of fluid during washing caused considerable loss of nitratase activity This inactivation was variable in extent and not nearly so marked in unadapted cells A similar sort of "dilution phenomenon" has been reported in the case of other enzyme systems and was found for the nitratase *adaptation* process (Pollock and Wainwright, 1948), although in the latter case it first became apparent at a higher concentration of cells than for the nitratase enzyme system itself

The first problem was fairly easily overcome by the addition of M/2200 oxine to the growing culture Fig 1 illustrates an experiment in which two lots of cells were grown in 10 ml of tetrathionate medium in Thunberg tubes containing 1 ml of water and 1 ml of M/200 oxine, respectively, in the stoppers After 4 hours' growth the contents of the stopper were tipped into the main tube Growth continued in the culture to which water had been added (Curve B), while it was almost completely and immediately arrested by the oxine (Curve A) for at least an hour Nitratase adaptation was also completely inhibited by this concentration of oxine, but the enzyme itself was quite unaffected After addition of the oxine, the cells were washed once with water before being tested for nitratase activity It was confirmed that the nitratase activity of cells treated in this way with oxine did not differ significantly from that of cells similarly treated but without oxine, and it seems reasonable to assume that the nitratase activity of samples from a growing culture measured in this way must correspond very closely with the true enzyme activity of the cells in the growth medium at the time of adding oxine

The second difficulty was largely, though not completely, overcome by the addition of M/800 freshly prepared $FeSO_4$ to the suspension mixture during measurement of nitratase activity Table II shows that the loss of activity on dilution and/or washing was very considerably reduced by Fe^{++}, but that some stimulation of nitratase in unwashed, undiluted cells (Expt 1 and 2)

TABLE II —*Inactivation of the Nitratase of Nitrate-Adapted Cells caused by Washing at Low Concentration, and its Reversal by Fe^{++}*

	Unwashed Cells			Cells twice washed at a strength of 50 µg /ml		
		Nitratase activities			Nitratase activities	
Expt No	Final conc cells µg /ml	Without Fe^{++}	With Fe^{++}	Final conc cells µg /ml	Without Fe^{++}	With Fe^{++}
1	200	3 8	4 4	50	1 9	3 1
2	200	4 4	4 8	28	1 1	5 3
3	48	1 6	3 7	36	0 3	2 2

occurred with Fe^{++}, and the extent of reactivation was variable, being complete in Expt 2, but only 60 per cent in Expt 3   Repeated attempts to obtain more consistent figures were not successful, and it must be admitted that results given for nitratase activity shortly after inoculation, where cell concentrations used in the enzyme assay were low (usually about 100 μg /ml), may be subject to some error   Nevertheless, it is not felt that such possible inaccuracies seriously affect the general picture given by curves relating growth and enzyme activity, and these experiments have been repeated many times with very similar results
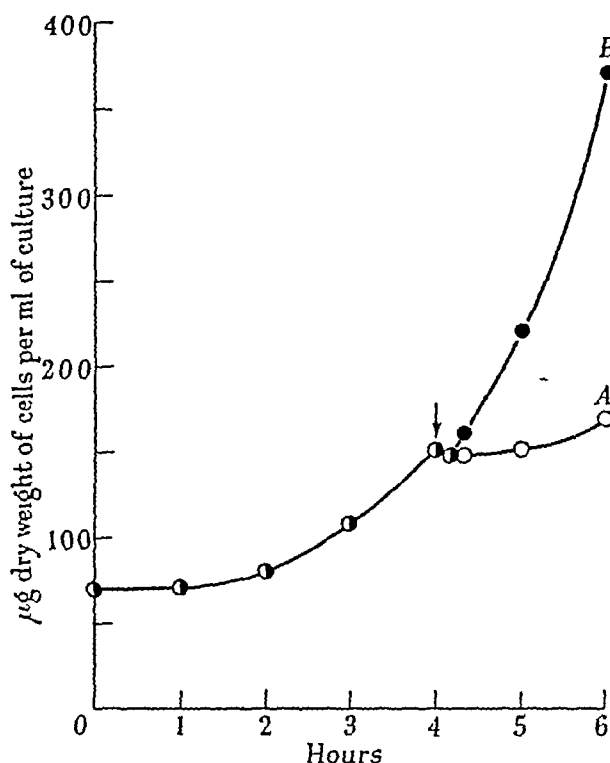
FIG 1 —Effect of M/2200 oxine on growth of " 1433 " in tetrathionate medium   Additions were made to the culture at the point indicated by the arrow (see text)   A, Oxine added B, Water added

A slight precipitate formed on addition of the Fe^{++}, but the estimation of nitrite was not interfered with in any way   It appears likely that this " dilution " phenomenon is due to a more or less completely reversible oxidation of some component in the system , similar but less satisfactory results could be obtained with other reducing agents such as cysteine, and glutathione

Results have been summarized in Fig 2 and 3, showing the relation between nitratase activity per mg dry weight of cells, total nitratase of the culture and growth, expressed as dry weight, of adapted and unadapted cells growing in the different media   For comparison, results obtained in a nitrate-containing medium are shown in Fig 4   The period covered by these studies allows for a maximum of only a 10-fold increase in cell mass—probably about three cell generations , but even this relatively small amount of growth is sufficient for the nitratase activity of previously adapted cells to have approached that of the unadapted

cells   There is no significant difference between growth rates of adapted and unadapted cells in media without nitrate, although, of course, in the nitrate medium, nitrate-adapted cells start growing appreciably sooner, as shown previously (Pollock and Wainwright, 1948)
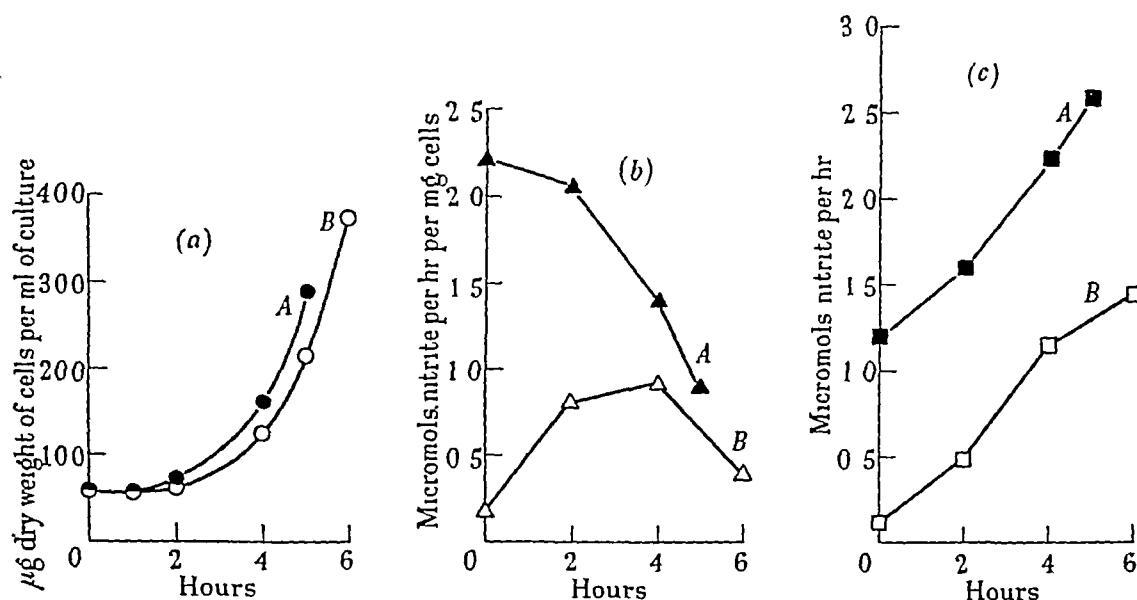
FIG 2 —Variation of nitratase activity of nitrate adapted and unadapted cells during growth in tetrathionate medium   A, Adapted cells   B, Unadapted cells
    (a) Growth curves
    (b) Nitratase activity of cells
    (c) Total nitratase activity of culture
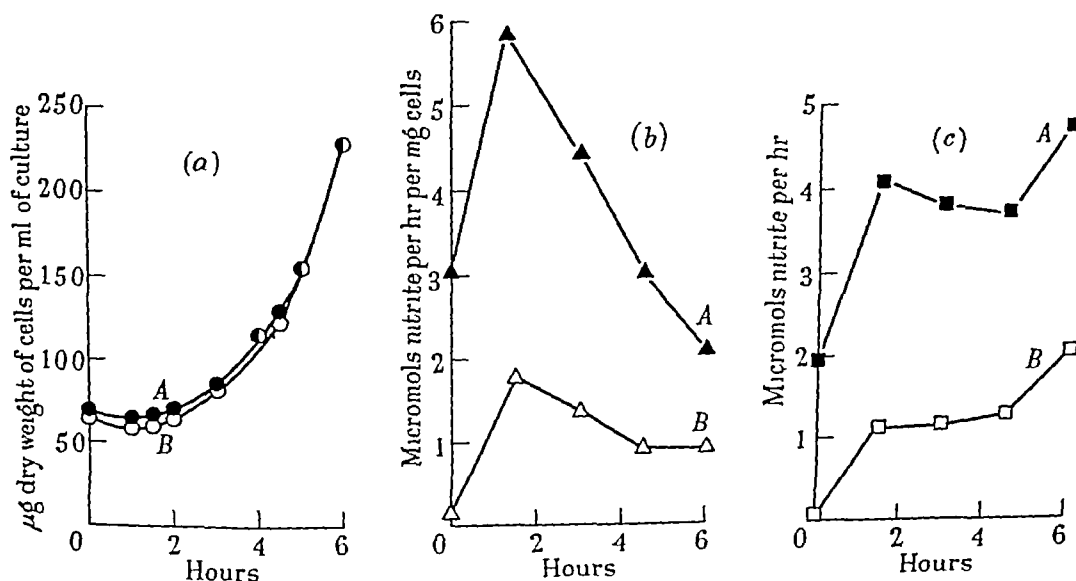
FIG 3 —Variation of nitratase activity of nitrate adapted and unadapted cells during growth in fumarate medium   A, Adapted cells   B, Unadapted cells
    (a) Growth curves
    (b) Nitratase activity of cells
    (c) Total nitratase activity of culture

It will be noted that there is quite a considerable, though variable, amount of " basal " nitratase formed by unadapted cells growing without nitrate, but that the excess total nitratase of the adapted cells over that of the unadapted cells remains very roughly the same at all stages of growth, and corresponds to
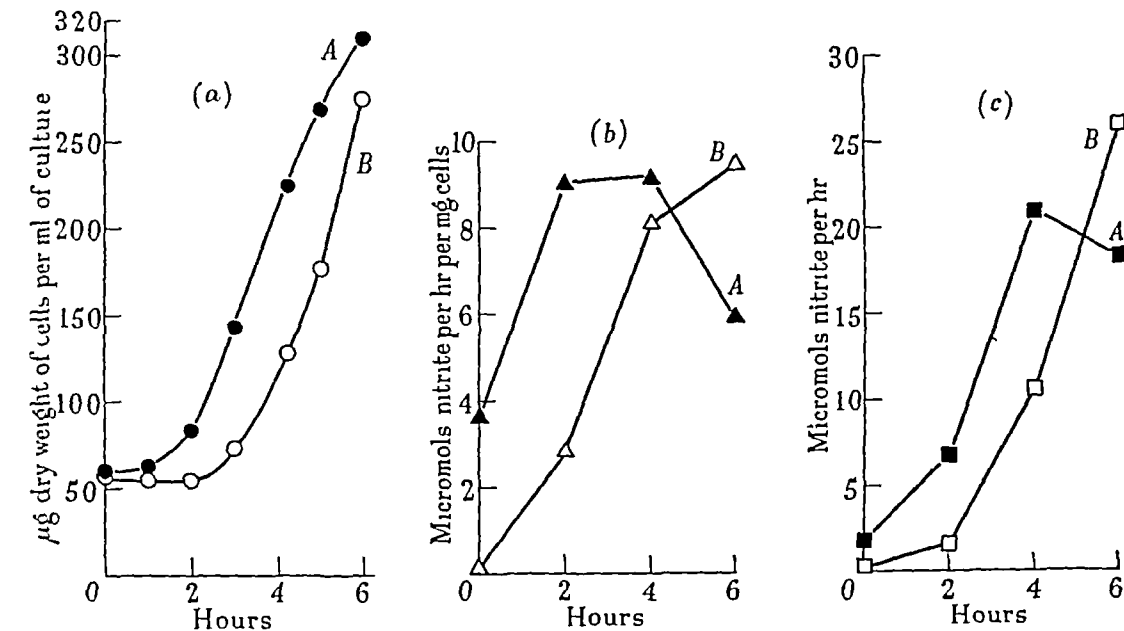
Fig 4 —Variation of nitratase activity of nitrate adapted and unadapted cells during growth
in nitrate medium   A, Adapted cells   B, Unadapted cells
  (a) Growth curves
  (b) Nitratase activity of cells
  (c) Total nitratase activity of culture
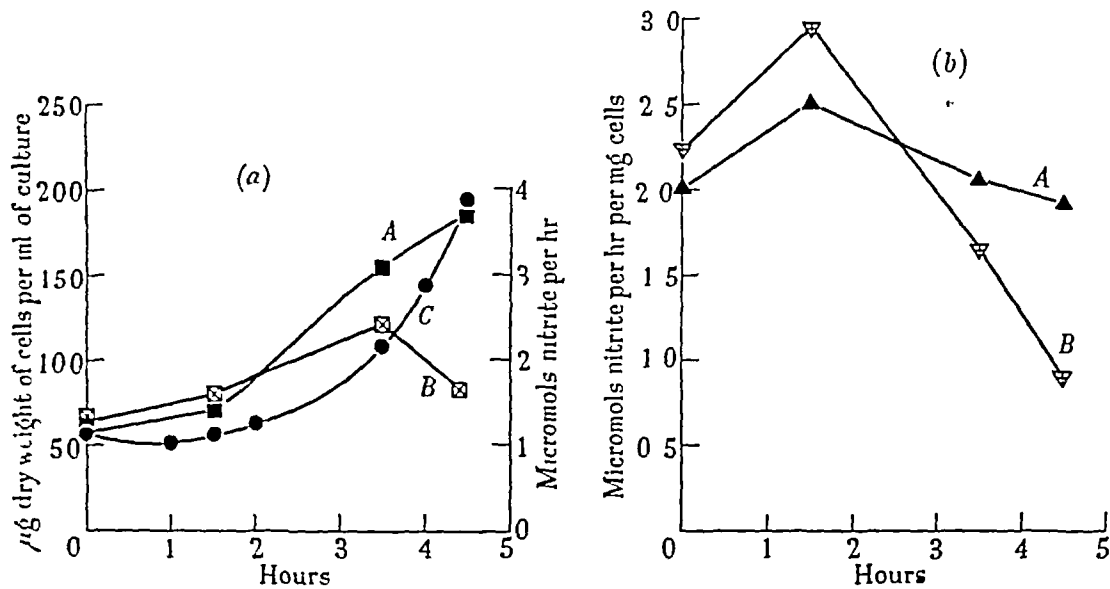
Fig 5 —Variation of nitratase activity of nitrate adapted cells during growth in tetra-
thionate medium as measured with different H donors
    (a) Total nitratase activities of the culture measured with formate (A) and lactate (B)
c, growth curve
    (b) Nitratase activities of the cells measured with formate (A) and with lactate (B)

the amount of preformed enzyme with which the adapted cells had been endowed during their initial, previous adaptation

It is also clear that, in the nitrate medium, adaptation of the previously unadapted cells proceeds very rapidly, and that the relatively higher nitratase activity of the adapted cells is not maintained for very long   After 4 hours the activities have become more or less equalized   The fall in the nitratase activity of adapted cells after 6 hours is probably due to the known toxic effect of the high concentration of nitrite and other nitrate reduction products which are formed , continued incubation of the previously unadapted cells produces a similar fall, not shown in this experiment   The further adaptation of previously adapted cells when grown with nitrate is simply a reflection of the difficulty of achieving maximal adaptation merely by treatment of washed cells with glucose and nitrate without growth   Growth with fumarate gives a similar result to that with tetrathionate, the only difference being a greater initial production of " basal " nitratase in both adapted and unadapted cells

It might legitimately be objected that in determinations of activities of complex enzyme systems such as nitratase, different results might be obtained according to what H-donor was used   However, in an experiment illustrated by Fig 5, nitratase activities of adapted cells have been followed during growth in the tetrathionate medium in parallel for a direct comparison between formate and lactate as H-donors   There is an unexplained drop in the total nitratase, measured with lactate, after $4\frac{1}{2}$ hours, and there is a bigger fall in nitratase activity during the whole period when using lactate than when using formate   On the whole, however, the differences are relatively slight, and the same general picture is shown whichever H-donor is used for the nitratase assay

DISCUSSION

These results show

(a) That " basal " nitratase formed by cells on growth without nitrate is subject to considerable variation

(b) That preformed nitratase of adapted cells growing in the absence of nitrate remains apparently unmodified at least over a period during which cell mass has increased 8-fold

Little comment is desirable on (a), especially since this will form the subject of further work at a later date   It seems likely that most adaptive enzymes are formed, at least to a slight extent, in the absence of substrate   Even in the case of yeast galactozymase—not normally detectable except after adaptation in the presence of galactose—Spiegelman and Reiner (1947) have shown that the enzyme can in fact increase under certain conditions in the absence of the substrate   The amount of basal enzyme formed varies from one strain of micro-organism to another, and might be expected to depend as well, to some extent, upon non-specific environmental factors

With regard to (b), the inactivation of the nitratase system during washing and dilution of the cells has led to some inaccuracy, particularly during the early part of growth   Nevertheless, the results are clearly quite different from those that might be expected were some sort of interaction between adaptive enzymes, as reported by Spiegelman and Dunn (1947) for yeast galactozymase and malto-zymase, occurring   Preformed nitratase remains apparently unmodified while the bacterial protoplasm increases, and the nitratase activity per cell approaches

the normal unadapted level   Still less is there any suggestion that treatment with nitrate has conferred on the cells some intrinsically increased ability to produce nitratase   The increase in total nitratase of the culture of adapted cells is no more than that which occurs with unadapted cells forming the usual amount of " basal " enzyme

SUMMARY

Nitratase activities of nitrate-adapted cells of the coliform bacterium " 1433 " growing in a medium without nitrate have been studied

This preformed nitratase of adapted cells remains unmodified during growth of the rest of the bacterial protoplasm , so that the enzyme activity per cell gradually falls, during growth, to its normal preadaptive level, by a simple process of " dilution-out "

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THE " LEWIS " BLOOD GROUP CHARACTERS OF ERYTHROCYTES AND BODY-FLUIDS

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A NEW blood group character designated " Lewis " was first described by Mourant (1946) who found that the red blood cells of about 25 per cent of English people were agglutinated by anti-Lewis sera and that the agglutination was independent of the ABO, MN, Rh, P, Lutheran and Kell systems   Jakobowicz, Simmons and Bryce (1947) described a further example of such a serum and Dr Mourant informs us that he has had several sera of similar character sent to him from the Regional Blood Transfusion Laboratories in this country

Andresen (1947) reported that he, and independently Dr Freisleben, had encountered several sera which agglutinated the red cells of 21 per cent of adult Danes   It is now known that the antibody in these sera is identical with the antibody in Mourant's original sera   Andresen (1947) observed that his sera agglutinated the erythrocytes of about 70 per cent of Danish children who were less than 7 months old, and that parents, both of whose red cells failed to agglutinate with these sera could have children whose erythrocytes were agglutinated   He concluded that the agglutinability of the erythrocytes of adults behaves as a recessive Mendelian character, that is, adult persons whose red cells possess this agglutinability are genetically homozygous

In a further paper Andresen (1948) described an additional antibody which in most of its reactions showed an antithetical relationship to the original Lewis antibody The red cells of 6 per cent of Group O and about 37 per cent of Group A_1 persons, however, were not agglutinated by either of these sera Andresen considered that the gene A_1 could have an epistatic action on the agglutinogen defined by this additional antibody

A revised notation to cover the recent developments in the " Lewis " blood group system has recently been proposed by Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles and Race (1949) and has been used throughout the present paper The two known Lewis genes recognized by Mourant and by Andresen respectively are now designated Le^a and Le^b, the corresponding antibodies as anti-Le^a and anti-Le^b and the gene products as Le^a-substance and Le^b-substance If the antiserum, anti-Le^a, is used alone in agglutination tests the phenotype of the cells which react is defined as Le(a +), those not agglutinated, as Le(a —)

Our immediate interest in the Lewis blood group system arose from the observation that in a group of 62 persons all those whose red cells were agglutinated by anti-Le^a serum and who were presumably of the genotype Le^aLe^a, were nonsecretors of their corresponding A-, B-, H-substances The results of the complete investigation, a preliminary account of which has been published by Grubb (1948), are now given

<div style="text-align:center">EXPERIMENTAL</div>

<div style="text-align:center">*Materials and Methods*</div>

Test sera

Anti-Le^a sera from nine persons, including the original serum from Mrs Lewis, were used Eight of the sera were obtained through the courtesy of Dr Mourant, who had identified them as anti-Le^a sera Two of these sera were from persons of Group AB The anti-Le^a serum " Hughes," which was used throughout the work described in this paper and was kindly supplied by Miss Boorman, Miss Dodd and Mr Gilbey, of the National Blood Transfusion Service, came from a person with the following group characters A_2, Le(a —), secretor of A- and H-substances, CDe/CDe, Kell-ve, Lu(a —) This serum is referred to as the " Standard " anti-Le^a serum, and its anti-Le^a character was fully confirmed by us in tests involving a panel of known Le(a +) (9) and Le (a —) (27) bloods A small amount of a serum " Buckstein " which was considered to be identical with Andresen's second type of anti-Lewis serum, now designated as Le^b antibody, was also available

Test-cells

Standard test-cells were Group O cells selected as representing the genotypes Le^aLe^a and Le^aLe^b Cells considered to represent the genotype Le^aLe^a were strongly agglutinated by the standard serum, were not agglutinated by anti-Le^b and were obtained from a person whose saliva inhibited completely at a dilution of 1 100 the standard dose of anti-Le^a serum Cells which were not agglutinated by anti-Le^a serum, were agglutinated by anti-Le^b serum and came from a person one parent of whom was of the phenotype Le(a +) were accepted as being heterozygous Le^aLe^b cells The saliva of the heterozygous person showed some power to inhibit anti-Le^a agglutinins

Agglutination and inhibition tests

All agglutination and inhibition. tests were carried out with once-washed, freshly-prepared erythrocyte suspensions   The degree of agglutination was observed microscopically after smearing a drop of the test material, serum-erythrocyte suspension, on a glass slide   Agglutinations were read after the tests had stood for at least 2 hours at 14–16°   It was observed that after washing the Le(a +) cells six times with saline they were agglutinated by the " standard " anti-Le^a serum to a much lower titre (4) than was normally observed with once-washed cells (16)

The influence of temperature on the degree of agglutination brought about by the " standard " anti-Le^a serum on Group O erythrocytes believed to represent cells of the genotypes Le^{a}Le^a, Le^{a}Le^b and of the phenotype Le(a — b —) was observed   The serum failed to agglutinate any of the cells at 37° but reacted weakly with Le^{a}Le^a cells at 25° and more strongly (titre 16) at 16°   The Le^{a}Le^b cells were not agglutinated at these temperatures but reacted weakly at 10° Cells of the phenotype Le(a — b —) remained unagglutinated at 10°

Owing to the strictly limited amount of the anti-Le^a and anti-Le^b sera available the agglutination technique described by Mourant (1948) for Rh agglutination was employed   The inhibition tests were carried out as follows   The serum (0 01 ml) was filled into a series of tubes (5 × 50 mm) by means of a Pasteur pipette graduated at 0 01 ml and an equal volume of the appropriately-diluted test-fluid was added   The contents were mixed by gentle tapping and allowed to stand at 14–16° for 30 minutes   The erythrocyte suspension (0 01 ml of a 1–2 per cent red cell suspension) was then added to each tube and the contents mixed   After standing for at least 2 hours at 14–16° the tubes were examined for agglutination

The tests used to determine the presence or absence of A and B substances in the secretions and body-fluids were performed as described by Morgan and King (1943)   The detection of the so-called " O " substance (the H-substance of Morgan and Watkins, 1948) was made by means of an anti-H serum engendered in the rabbit against an artificial antigen built up from purified H-substance and the conjugated protein of the O antigen of *Shigella shigae*   The H activity of the materials was also established by similar inhibition tests which used a serum from the eel *Anguilla vulgaris* (Jonsson, 1944 , Grubb, 1949), which serum, at a dilution of 1 100, agglutinated O cells strongly but was without action on A$_1$B cells

The pig stomach extracts examined were prepared by autolysis of individual stomach linings at pH 3–4 (Aminoff, Morgan and Watkins, 1946 , Bendich, Kabat and Bezer, 1946 , Chadwick, Smith, Annison and Morgan, 1949)   The human ovarian cyst fluids were from women belonging to Groups A, B, O and AB   Fluids from secretors and nonsecretors within the ABO classification were investigated (Morgan and van Heyningen, 1944)

The preparations of A- and H-substances were obtained by methods already described (Morgan and King, 1943 , King and Morgan, 1944 , Morgan and Waddell 1945) and purified until the materials were essentially homogeneous by physical chemical and immunological tests   Full details for the preparation of these materials will be given elsewhere   The A-substance contained 5 7 per cent N and 9 1 per cent CO CH$_3$, and showed a *dextro* rotation, $[\alpha] + \dfrac{16° \pm 3}{5461}$ The examination of the products of hydrolysis with 0 5 N

HCl revealed that the A-substance contained 37–38 per cent hexosamine (as base) and gave rise to 56 per cent reducing sugars measured as glucose   The α-amino acid N, 2 1 per cent, comprised 38 per cent of the total N   The H-substance contained 5 4 per cent N and was *laevo*-rotatory $[\sigma]$–30°   The material gave rise to 35 per cent hexosamine (as base) and 53 per cent reducing sugars after hydrolysis at 100° with 0 5 N HCl   A mucoid material, obtained from an ovarian cyst fluid which was devoid of A, B, H, Le^a or Le^b activity was used together with dextran, as a control substance, in inhibition tests   The mucoid contained 5 9 per cent N and showed no significant rotation

RESULTS

The examination of 212 erythrocyte specimens from nonrelated adults living in South-East England showed that 47 (22 2 per cent) of them were Le (a +)   The salivas of the same persons were tested for the secretion of A-, B- and H-substances and the result showed that 49 (23 1 per cent) were non-secretors of these substances   There was some selection within the ABO classification and too few Group O persons were included

A rather larger group of similar results representing all specimens examined is set out in Table I, from which it will be seen that of 222 individuals investigated, 57 were Le(a +) and nonsecretors of the A-, B- and H-substances   The remainder (165) were Le(a —) and of these 163 secreted the appropriate A-, B- and H-factors   In this group, therefore, there are two persons who are Le(a —) and nonsecretors of A-, B and H-substances

TABLE I —*Correlation Between Le^a Blood Groups and Secretor Character Within the ABO Classification* *

	O	A$_1$	A$_2$	A	B	AB	Total
Erythrocytes Le(a +) A,B,H. nonsecretors	23	11	6	10	5	2	57
Erythrocytes Le(a —) A,B,H secretors	47	45	14	30	18	9	163
Erythrocytes Le(a +) A,B,H secretors	0	0	0	0	0	0	0
Erythrocytes Le(a —) A,B,H nonsecretors	0	1	1	0	0	0	2
	70	57	21	40	23	11	222

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* This table includes the results recorded earlier (Grubb, 1948)

The secretion of a water-soluble, serologically specific Le^a-substance was investigated by means of inhibition tests made with saliva at three dilutions (1 5, 1 25 and 1 100) using the standard anti-Le^a serum ' Hughes, titre 1 16 and Le^{a}Le^a test-cells   The first 10 saliva specimens examined were investigated with two additional anti-Le^a sera using test cells obtained from four different Le (a +) persons   The only difference observed was that a weaker serum gave a higher inhibition titre, a result which could have been anticipated   A summary of the results obtained with salivas from 50 persons are set out in Table II from

which it is evident that all persons so far investigated whose red cells are Le (a +) secrete Le^a-substance in the saliva, whereas only some of the persons whose red cells are Le(a —) do-so   The salivas of persons from the former group are on the whole more potent than the active salivas obtained from the latter group   A specimen of gastric juice obtained from a person of phenotype Le (a +) contained Le^a-substance

TABLE II —*The Results of Inhibition Tests Using Saliva and Anti-Le^a Serum*

Saliva from persons with	Number of specimens	Percentage of salivas inhibiting anti Le^a agglutination Saliva dilution		
		1 5	1 25	1 100
Le(a —) red cells	50	84	40	0
Le(a +) red cells	30	100	100	90

The examination of the distribution of the water-soluble Le^a-substance in the body-fluids was extended to a large number of ovarian cyst fluids and the results of inhibition tests, which are set out in Table III, showed that some cyst fluids contained relatively large amounts of Le^a-substance   The cyst fluids were collected earlier for another purpose and it is, unfortunately, not known whether the red cells of the persons concerned were Le(a +) or Le(a —)   It will be seen, however, that against a strongly-agglutinating dose of the " standard " serum some cyst fluids could be diluted many thousands of times, and were then able to inhibit completely the agglutinating action of the anti-Le^a serum   One cyst fluid, "UN", showed no significant amounts of A-, B- and H- substances but was rich in Le^a-substance   Preparations of purified and essentially homogenous human Group A-substance and H-substance of both human and animal origin show no significant power to inhibit the agglutination of Group O, Le (a +) erythrocytes by anti-Le^a serum

TABLE III —*Showing the Inhibition of Anti-Le^a Serum by Ovarian Cyst Fluids*

Cyst fluid	Group	ABH Secretor (S) or non secretor (s)	Dilution of cyst fluid inhibiting the agglutination of Le(a +) erythrocytes by anti Le^a serum					
			1 10	1 100	1 1000	1 3000	1 12,000	1 50,000
67	O	S	0	0	0	0	0	0
115	B	S	0	0	0	0	0	0
2S	B	s	0	0	0	1	2	2
105	A	S	0	0	0	0	1	2
111	A$_1$B	S	0	0	1	2	3	3
117	A$_1$B	S	0	1	2	2	3	3
29	A$_1$B	s	3	3	3	3	3	3
UN	—	s	0	0	0	0	0	0
Saline	—	—	3	3	3	3	3	3

Degrees of agglutination   O, no agglutination , 1, a few groups of 2–3 cells , 2, larger groups with many free cells , 3, many small clumps

The results of a few similar tests set up to detect the secretion of a soluble Le^b-substance are given in Table IV   A specimen of mucoid obtained from an

ovarian cyst fluid which showed Le^b activity inhibited a strongly agglutinating dose of anti-Le^b serum at a dilution of 1 2 × 10^6 It may be concluded, therefore, that this gene product is also secreted in a water-soluble form

TABLE IV —*Showing the Inhibition of Anti-Le^b-Serum by Saliva*

		Final dilution of saliva inhibiting anti Le^b serum		
Donor's red cell phenotype		1 3	1 15	1 75
Le(a +)	. .	3	3	3
Le(a +)		3	3	3
Le(a — b +)		0	0	0
Le(a — b +)		0	0	0
Le(a — b —)		3	3	3
Le(a — b —)		3	3	3

Degrees of agglutination as in Table III

Of 165 persons whose erythrocytes failed to react with anti-Le^a serum, 163 secreted the appropriate A, B, and H factors in a water-soluble form, whereas 2, whose blood belonged to the Groups A$_1$ and A$_2$ respectively, failed to do so The reactions of the blood and saliva specimens from these individuals were confirmed on fresh samples and there appears to be little doubt that these persons are exceptions to the rule that individuals whose red cells are not agglutinated by anti-Le^a serum secrete the appropriate A-, B-, and H-substances

The erythrocytes of one of the individuals (M R , Group A$_1$) were not agglutinated by any of our panel of anti-Le^a or anti-Le^b sera and her saliva failed to inhibit the agglutination of A$_2$ cells by natural (human) anti-A serum or the haemolysis of sheep cells by rabbit anti-sheep cell serum The saliva from these two individuals contained neither Le^a- nor Le^b-substance The red cells of both persons were of the phenotype Le(a — b —)

MISCELLANEOUS OBSERVATIONS

During the investigation a number of additional observations were made which seem worth recording For example, no natural anti-Le^a agglutinins were detected in 21 normal rabbit or in 14 normal chicken sera Specific Le^a-substance was not present in the mucoid materials prepared from 50 different pig stomach linings by acid autolysis and it would appear that preparations of A- and H-substances from this source can, therefore, be used for the neutralization of anti-A and anti-H agglutinins which may be present in human anti-Le^a sera A mucoid material prepared from sheep submandibular gland (McCrea, unpublished) which showed considerable activity as a receptor substance for heated influenza virus likewise showed no Le^a activity Anti-Le^a agglutinins were not absorbed by specimens of red cells from six rabbits and were not neutralized by the specific polysaccharide of Pneumococcus, Type XIV, or by the purified O antigen of *Shigella shigae*

The serum from a person of genotype Le^{a}Le^a neutralized a strongly agglutinating dose of anti-Le^a serum although at a dilution of 1 2 only, whereas the serum from a person of the phenotype Le(a — b —) was without power to inhibit Le^a-substance is, therefore, most probably present in low concentration in the serum of persons whose red cells are agglutinated by anti-Le^a serum

The enzymic inactivation of the specific Le^a- and Le^b-substances

The action of enzyme preparations obtained from culture filtrates of certain strains of *Cl welchii* (Type B) on purified preparations of the Le^a-substance was examined It had already been shown that these enzymic preparations inactivate serologically the A-, B- and H-substances (Morgan, 1946 Stack and Morgan, 1949) and that the enzymic activities could to some extent be differentiated by heating the enzyme preparation at 56° for 1 hour whereby the activity against the group substances A and B is lost The heated solutions, however, are able to destroy the serological character of the H-substance The Le^a-substance (1 0 ml of 0 1 per cent solution in buffer at pH 6 4) was mixed with an equal volume of the enzyme solution and incubated at 37° for 2 hours in the presence of toluene The enzyme solution used was (*a*) unheated, (*b*) heated at 56° for 1 hour, and (*c*) heated at 100° for 15 minutes After incubation the enzyme-substrate solutions were heated at 100° for 10 min to inactivate an enzyme present which brings about a sensitization of the red cells used in the agglutination-inhibition test performed subsequently to measure the degree of inactivation of the Le^a-substance The results of an experiment of this kind are given in Table V from which it will be seen that the Le^a-substance is rapidly destroyed, whereas the enzymic material, after heating for 1 hour at 56° is almost inactive Similar results were obtained with a partially purified specimen of Le^b-substance

TABLE V —*Showing the Inactivation of Le^a-Substance by a Cl welchii (Type B) Enzyme Preparation*

	Dilution (× 10⁴) of Le^a substance inhibiting the agglutination of O, Le(a +) cells by anti Le^a serum								
Treatment of enzyme	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512
Unheated	0	0	1	2	3	3	3	3	3
Heated 56° 1 hr	0	0	0	0	0	1	2	3	3
,,　100° 15 min	0	0	0	0	0	0	1	2	3

Degrees of agglutination as in Table III

DISCUSSION

A close correlation of the Lewis blood-group character with the secretor-nonsecretor status within the ABO classification has already been recorded by Grubb (1948) in a preliminary communication involving the data obtained from a relatively small group of adults The results of the main investigation are communicated in this paper and confirm the earlier finding that persons whose red cells react with anti-Le^a serum are also nonsecretors of the A-, B- and H-substances More recently, Race, Sanger, Lawler and Bertinshaw (1949) have likewise fully confirmed this interesting relationship Furthermore, these authors showed that the data obtained from family studies are consistent with Andresen s view that adults whose red cells are agglutinated by anti-Le^a serum are genetically homozygous

The frequency of bloods in our material giving agglutination with anti-Le^a serum, 22 2 per cent, agrees closely with the figure 22 7 per cent found by

Race *et al* (1949) in a similar English population   The frequency of nonsecretors of A-, B- and H-substances, 23 per cent, found by us is of the same order as that reported for other populations   The corresponding figures are, for Germany, 22 0 per cent (Schiff), Denmark, 26 0 per cent (Hartmann, 1941) and New York, 18 per cent (Schiff see Wiener, 1946)

Le^a- and Le^b-substances are secreted in a water-soluble form as are the A-, B- and H-substances and, therefore, the terms secretor and nonsecretor should not be used without indicating clearly the group system involved   Le^a- and Le^b-substances resemble the A-, B- and H-substances in general physical and chemical properties   They are heat stable, water-soluble substances of mucoid nature and are readily decomposed by an enzyme in *Cl welchii* (Type B) culture filtrates   The Le^a-substance occurs in saliva, gastric juice, ovarian cyst fluids and serum   The presence of the Le^b-substance has not been tested for in gastric juice or serum but it has been shown to occur in a water-soluble form in saliva and ovarian cyst fluids

Le^a-substance was clearly demonstrable in the saliva from 30 persons whose red-cells were of the phenotype Le(a +)   The majority of salivas from persons whose erythrocytes were Le(a —) also contained Le^a-substance   These observations are very different from those encountered in the ABO system where, for example, every fifth person belonging to Group A does not secrete A-substance, and A-substance does not occur in the secretions of persons whose red cells fail to react with anti-A serum, that is in those persons who belong to Groups O or B

It seems as if all persons possessing the *Le^a* gene secrete Le^a-substance   One consequence of accepting Andresen's view that persons whose red cells are agglutinated by anti-Le^a serum are of the genotype Le^{a}Le^a, is that a majority of persons whose erythrocytes fail to react with anti-Le^a serum nevertheless possess an Le^a gene   It is not surprising, therefore, that the specific Le^a-substance may be encountered in the secretions of these persons   In secretions and body-fluids the Le^a-substance is in solution and its presence is demonstrated by means of the very sensitive agglutination-inhibition test   In red cells, on the other hand, the specific Le^a-substance is part of a fixed surface structure, the Le^a agglutinogen, and may be subject to the influence of co-existing heterologous determinant groupings and other unspecific factors which may combine to reduce the sensitivity of the agglutination test   The differences in substrata and technique may, therefore, conceivably make the whole difference between detection (in the saliva) and non-detection (in the erythrocytes) of a single dose of the Le^a-gene   Although it may now be accepted that the Le^a-substance occurs in the secretions of many persons whose red cells seem to lack the corresponding agglutinogen, it can be anticipated that the practical application of this knowledge, the demonstration of the presence of a single dose of the *Le^a* gene by means of inhibition tests using saliva, will be beset with difficulties   It is known that within the ABO system the differentiation of secretors from nonsecretors is in certain instances not very satisfactory (Hartmann, 1941) and the results of the present study indicate that the Le^a secretion-nonsecretion phenomenon represents more gradual and continuous variation than is met with in ABH secretion-nonsecretion   It is a matter of conjecture, therefore, whether some of the cases are to be classified as secretors or as nonsecretors of Le^a-substance

As the great majority of salivas obtained from persons whose red cells fail to react with anti-Le^a serum contain some specific Le^a-substance, only a minority

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of salivas could be used for the neutralization of anti-A or anti-B agglutinins in anti-Le^a sera

In our material all persons who possess Schiff's secretor gene, S, have red cells which are not agglutinated by anti-Le^a serum   There is, therefore, a close although inverse relationship between the Le^a and S genes   It is known from Andresen's results, however, that the majority of these bloods will react with anti-Le^b serum and, furthermore, it is probable that no bloods of the phenotype Le (a + b +) exist   It might be anticipated, therefore, that there is some kind of relationship between Le^b and S genes   Owing to the very limited amount of anti-Le^b serum available we have been forced to confine our tests to a few instances where the erythrocytes or secretions were deemed of special interest   It was thought earlier that a very close relationship might exist between H-substance and Le^b-substance and in consequence a number of specimens of H-substance of human origin were tested for their capacity to neutralize H and Le^b antibody and the two activities expressed as a ratio   The results showed that H-substance and Le^b-substance cannot be identical   Furthermore, we have encountered an individual of the phenotype O, Le(a — b —) who secretes H-substance but not Le^a or Le^b-substances   Dr Race informs us that he has encountered three persons with similar characters   The frequency of the occurrence of the phenotype Le(a — b —), secretor of A-, B- and H-substances, cannot be estimated on our material as the very limited amount of anti-Le^b serum available has precluded an adequate investigation for the presence of this gene product

From what is already known of the Lewis system it can be surmised that Le^b-substance and some or all of the A-, B-, H-substances will be frequently found together in the body-fluids and secretions

It is evident from the results given in Table I and from the findings of Race et al (1949) that the correlation between Le(a +) blood group character and secretor status within the ABO classification, is a very close one   On the basis of probability it would seem impossible that these results have arisen by chance   A simple rule seems to hold and may be expressed as   Adult persons with Le(a +) red cells are nonsecretors of A-, B- and H-substances and adult persons who possess Le(a —) red-cells are secretors of these substances   As a result of this relationship it follows that in adults a good estimate as to the secretor-nonsecretor status of an individual within the ABO classification can be made on the basis of a simple agglutination test with anti-Le^a serum   So far less than 1 per cent of individuals are known to be exceptions

Individuals whose red cells are agglutinated by anti-Le^a serum most probably possess the Le^a-gene in double dose and they are nonsecretors of the A-, B-, H-substances   Schiff and Sasaki (1932) have shown that persons who are non-secretors of the A-, B-, H-substances possess the gene s in double dose   Does the close correlation of Le(a +) red cell character and non-secretion of the A-, B-, H-substances indicate that the genes Le^a and s are identical?   We have observed a few individuals of the phenotype Le(a — b —) in whose saliva no A-, B-, H-, Le^a- or Le^b-substances were demonstrable   The absence of these gene products is not due to impaired health or old age and indeed the examination of 10 persons, within the age group 55–80, who were suffering from Addisonian anaemia and were thus nonsecretors of hydrochloric acid, revealed no exceptions to the rule that Le(a —) persons are secretors of the appropriate A-, B-, H-substance   It seems probable, therefore, that the phenotype Le

(a — b —) nonsecretor of A-, B-, H-substances is an accurate reflection of the genetical constitution of these individuals and the existence of such individuals makes it improbable that the genes Le^a and s are identical Is this an example of linkage in man? If Le^a and s genes are linked they must be very closely linked, otherwise crossing-over would have obliterated the Le(a +)-nonsecretor association in the general population

If the genes Le^a and s are linked, this linkage is so close that one might place these genes, and consequently the genes Le^b and S, in one genetic system Furthermore, the general similarity of the physical and chemical properties of the soluble products of the Le^a, Le^b and S genes might be an additional reason for considering that they belong to the same system

The Lewis system and its relationship to the secretor-nonsecretor phenomenon within the ABO classification cannot be discussed at length in this paper It is, nevertheless, worth considering briefly It is suggested that there are three allelomorphic pairs of genes, S and s, Le^a and Le^α, Le^b and Le^β, which occupy contiguous loci The genes Le^α and Le^β, are allelomorphic to the genes Le^a and Le^b respectively and are unknown at present The symbols Le^α and Le^β are used here solely to illustrate the hypothesis Such a system would be similar in many respects to the Rh system and of the eight possible combinations on a single chromosome, four only, $s\ Le^a\ Le^\beta$, $s\ Le^\alpha\ Le^\beta$, $S\ Le^\alpha\ Le^\beta$, $S\ Le^\alpha\ Le^b$, occur at all frequently in our material The data so far recorded would appear to fit a system of this kind which comprises a closely-packed constellation of three individual genes We are well aware of the tentative character of the hypothesis The predictions inherent in the scheme, however, are largely open to experimental proof

SUMMARY

1 The examination of blood and saliva of 212 persons has shown that 22 2 per cent are Le(a +) and that these individuals all fail to secrete A-, B- and H-substances There is, therefore, a very close association between Le (a +) red cell character and inability to secrete in terms of the A, B and H blood-group factors

2 Le^a- and Le^b-substances are present in secretions of persons of the appropriate genotype and show the same general physical and chemical characters as the A-, B- and H-substances

3 It seems probable that all persons possessing the Le^a gene secrete Le^a-substance

4 Some human ovarian cyst fluids are a convenient and potent source of the Le^a- and Le^b-substances

5 The specific Le^a- and Le^b-substances are inactivated by an enzyme present in *Cl welchii* (Type B) culture filtrates

6 Le^b- and H-substances are not identical

7 It is suggested that the Le^a, Le^b, S and s genes belong to one and the same system

8 Among 222 individuals, two nonsecretors of A-, B-, H-substances whose red cells were Le(a —) were found These two individuals were also nonsecretors of Le^a- and Le^b-substances and their red cells were Le(b—) A person of the phenotype O, Le(a — b —), who was a secretor of H-substance and a nonsecretor of Le^a- and Le^b-substances was also encountered The occurrence

of persons with these serological characters might be considered to indicate the existence in the Lewis system of additional, unknown genes

Our thanks are due to Dr R R Race and Dr Ruth Sanger for much helpful advice during the investigation and to the staff of the Regional Blood Transfusion Depot, Sutton, for a generous supply of bloods and salivas

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ALVEIN

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DURING a survey of the antibacterial properties of aerobic spore-forming bacilli, Gilliver (1949) found that a strain of *Bacillus alvei* (code number 642 in the collection of Dr Gibson of Edinburgh), when streaked on lemco agar, produced diffusible substances that inhibited the growth of a number of Gram-positive and Gram-negative bacteria This paper contains a preliminary account of one antibiotic, named alvein, which is formed by *Bacillus alvei* 642 in certain liquid media

Production

The production and purification of alvein were followed by the use of the cylinder plate method of assay (Heatley, 1944) *Staph aureus* (N C T C 6571) was used as a test organism, and a dry preparation of alvein hydrochloride was adopted as an arbitrary standard At a dilution of 1 in 10,000 the standard preparation produced inhibition zones of from 15 to 18 mm in diameter

Early attempts to produce the antibiotic by growing *B alvei* in stationary liquid culture met with little success Active material was formed in a lemco-peptone broth, but only when a surface growth of the bacillus was facilitated by some mechanical support, such as by pads of cotton wool floating in the medium The use of stationary cultures was abandoned when it was found that an antibiotic was readily formed by deep fermentation in a medium containing glucose, corn steep liquor and inorganic salts

Medium and conditions of culture

The organism was grown in deep aerated culture in the following medium

NaNO$_3$	3 0 g	FeSO$_4$	0 01 g
KH$_2$PO$_4$	1 0 g	Corn steep liquor	10 ml
KCl	0 5 g	Glucose	10 g
MgSO$_4$ 7H$_2$O	0 5 g		

made up to 1 litre with tap water and adjusted to pH 7 0 before autoclaving

The fermentation was carried out in 10 litre bottles containing 8 litres of medium One ml of sterile triamyl citrate per litre of medium was added, after autoclaving, to prevent frothing The inoculum was 200 ml of an aerated overnight culture in the same medium The bottles were placed in a water bath at 37° C and the cultures aerated through perforated glass bulbs at the rate of about 1 litre of air per litre of culture fluid per minute After from 40 to 48

hours the culture fluid was at pH 6 6–7 0 and its activity was approximately equivalent to that of a 1 in 80,000 solution of the standard alvein hydrochloride

In a medium containing lower concentrations of corn steep liquor and glucose the activity fell off rapidly after reaching its maximum   When higher concentrations of corn steep liquor and glucose were used, a large proportion of the activity appeared to be due to an antibiotic other than alvein, which was active against *Myco phlei* but not against *Staph aureus*

Extraction and purification

In early small-scale experiments the antibiotic was extracted directly from the culture fluid (adjusted to pH 9 0) by butanol   For dealing with larger quantities of fluid it was more convenient to use a process involving adsorption onto charcoal   The process was carried out in the following manner

The culture fluid was cleared, either by standing overnight or by centrifuging The supernatant liquor was brought to pH 7, and shaken with 0 5 per cent charcoal (Farnell grade 14 neutral) which adsorbed the active material   The latter was eluted by shaking the charcoal with a two-phase mixture of n-butanol and N/5 HCl (1 3), the total amount used being 1/10th of the volume of the original culture fluid   Most of the eluate was concentrated in the butanol layer It was transferred to aqueous solution by mixing the butanol with one volume of ether, shaking the mixture with one volume of distilled water, and adjusting the pH to 2 0   The resulting aqueous solution was neutralized and filtered

A two-phase system of butanol and dilute HCl had previously proved useful for eluting the antibiotic ayfivin from charcoal (Arriagada, Abraham, Sharp, Savage, Heatley and Sharp, 1949)   Butanol is adsorbed on the charcoal and displaces the antibiotic, which partly enters the aqueous phase   In the case of alvein the antibiotic then passes largely into the layer of remaining butanol, the final system consisting of three phases   The method presumably depends on the reduction in concentration of the desorbed substance in the eluting solvent which results from the entry of the substance into the aqueous phase

Steenberg (1941) first described a procedure for desorption from charcoal which involved alternate treatments of the charcoal with an organic solvent, such as benzene, and water   The adsorbed substance was insoluble in the organic solvent but was displaced by it from the charcoal and could then be removed by water   The use of benzene and an aqueous phase was not successful with ayfivin   The efficiency of butanol and dilute HCl for eluting ayfivin, laterosporin (Barnes 1949) and alvein may be associated with the significant solubility of these antibiotics in butanol

After extraction from butanol, alvein was precipitated from aqueous solution by the addition of an excess of saturated picric acid at 4° C   The picrate was dried in a vacuum desiccator and then converted to the hydrochloride by stirring with ethyl alcohol containing 5 per cent N HCl   Alvein hydrochloride was precipitated from the acid-alcohol extracts by the addition of an excess of dry ether The precipitate was washed with ether and dried *in vacuo*   The yield was about 3–4 mg per litre of culture fluid and represented a recovery of 20–30 per cent of that present in the original culture fluid

The properties of alvein which are recorded in the following sections refer to material obtained in this manner

Further purification

To obtain some idea of the homogeneity of this preparation an eight tube counter-current distribution (Craig, Golumbic, Mighton and Titus, 1945) was carried out with 20 mg of active material Isobutanol-phenol (1 1) and 0 1 N aqueous acetic acid were used as the two phases and the total volume of solvents in each tube was 2 ml The overall partition coefficient of the antibacterial activity in the system was approximately one After the distribution, Tube 0 contained 6 5 per cent of the material, but no activity, while Tube 8 contained 5 6 per cent of the material and about the same proportion of activity The amounts of material and activity in Tubes 2–7 approximated to those expected if these tubes contained a single active substance with a partition coefficient of 1 28 It was thus possible that the preparation contained less than 10 per cent of inactive material and that the major portion of the activity was associated with a single substance, although a second active substance was present in a proportion of about 5 per cent

Chemical Properties

The preparation of alvein hydrochloride gave the following values on elementary analysis C, 49 0 , H, 8 7 , N, 12 9 , S, 1 2 , Cl, 8 9

The hydrochloride was easily soluble in ethanol and in water, but insoluble in acetone, chloroform or ether A solution in water had a pH of about 3 8 As the pH was raised by the addition of sodium hydroxide the substance became much less soluble Precipitation began when the pH approached 7 and increased as it was raised further to 9 The precipitate dissolved on addition of ethanol

On shaking a solution of alvein hydrochloride in aqueous alcohol with a suspension of silver oxide, or on adding an amount of sodium hydroxide equivalent to the chloride present, the solution became alkaline to phenolphthalein, indicating that the free substance was a strong base

Alvein was extracted by butanol from solution in water at pH 9 It was not removed by butanol from a solution in water at pH 3, but was readily extracted by phenol The presence of small amounts of sodium chloride greatly affected the partition coefficient of alvein between butanol and an aqueous solution at pH 3 One per cent sodium chloride caused most of the antibiotic to enter the butanol phase, and 0 1 per cent sodium chloride had a significant effect This action of salt was presumably responsible for the fact that after elution from charcoal under acid conditions the antibiotic was found largely in the butanol layer

In aqueous solution at room temperature alvein lost no measurable activity in two hours at pH 2, 7 or 9, but its activity was reduced in 0 1 N or 0 5 N HCl It retained its activity for one hour in boiling aqueous solution at pH 2, but in a similar solution at pH 9 half its activity was lost The activity of an aqueous solution of alvein (0 2 mg per ml) was reduced to about one eighth of its original value after incubation for 4 hours with commercial trypsin (1 mg per ml) at pH 8 Incubation with 50 per cent horse serum resulted in very little loss in activity

Alvein gave a positive Sakaguchi reaction for arginine, but negative colour reactions for tyrosine and tryptophane After hydrolysis for 20 hours with 6 N HCl at 105° C , the product gave a strong ninhydrin reaction The colour density yielded by a given weight of alvein hydrochloride, determined by the

method of Moore and Stein (1948), was almost identical with that given by the same weight of leucine   Analysis of the hydrolysate by paper chromatography (Consden, Gordon and Martin, 1944) indicated the presence of cystine, lysine, serine, threonine, alanine, valine and leucine   Lysine and leucine appeared to be present in large amounts   There was no indication of the presence of glutamic acid or aspartic acid

The preparation thus behaved as though it consisted mainly of a strongly basic polypeptide

Biological Activity

Antibacterial activity

The activity of alvein against a number of bacteria was determined by a serial dilution method in nutrient broth   The solutions of alvein hydrochloride were sterilized by filtration through a sintered glass bacteriological filter, and diluted in a two-fold series   Ten per cent of serum was present in the tests with *Str pyogenes* and *C diphtheriae*   The tubes were inoculated from overnight broth cultures of the test organisms, and the results were read after overnight incubation, the end-point being taken as that concentration of alvein which completely prevented visible growth   Further incubation for one day did not decrease the inhibitory level more than two-fold

The test with *Myco tuberculosis* was kindly performed by Dr A Q Wells The organism was cultured in Dubos' medium containing 10-fold dilutions of alvein, the inoculum being 0 1 ml of a 7-day culture in Dubos' medium   The results were read at 4 days and 11 days

The results obtained are shown in Table I

TABLE I —*Antibacterial Activity*

Test organism	End point at 24 hours 1 part alvein hydrochloride in x thousands
Staph aureus	512
Str pyogenes (C N 10)	128
C diphtheriae var *gravis*	128
C xerosis	8,000
Bact coli	32
Ps pyocyanea	16
B anthracis	512
Myco phlei	32,000
Myco tuberculosis (P N)	100 (4 days) 10 (11 days)

Attempts to increase the resistance of *Staph aureus* by growing it in the presence of sub-inhibitory amounts of alvein were unsuccessful   The action of alvein on *Myco tuberculosis* appeared to be bactericidal

Haemolytic activity —Alvein is haemolytic   Its action on human erythrocytes at 30° C was measured quantitatively in a photoelectric apparatus designed by Dr W E van Heyningen, to whom we are indebted for collaboration   At a dilution of 1 in 5 000, alvein produced 78 per cent haemolysis of a 0 4 per cent suspension of human red cells in 10 seconds   At 1 in 15,000 it produced 22 per cent haemolysis under the same conditions   After the first rapid change, subsequent haemolysis was relatively very slow

Bernheimer (1947) found that tyrocidine, in contrast to certain bacterial haemolysins, caused a rapid haemolysis which was followed by a slow haemolysis at a constant rate The phenomenon appeared to be due to inhibition of the action of tyrocidine by the products of haemolysis Although the haemolytic action of alvein resembles that of tyrocidine the two substances are quite distinct in other properties

Other Antibacterial Substances Produced by B alvei, 642

At least one antibacterial substance other than alvein appeared to be produced by *B alvei* in Czapek-Dox broth The evidence for an additional substance depends on the following observations

(1) When samples of culture fluid were incubated with commercial trypsin, their activity against *Staph aureus* and *C xerosis* was reduced to 1/8th–1/16th of the original value This reduction could be accounted for by the effect of trypsin on the alvein present in the fluid The activity against *Myco phlei*, however, was reduced only to one half

(2) The recovery of activity from the culture fluid was only about 10 per cent when *Myco phlei* was used as test organism, whereas it was 20–30 per cent when *Staph aureus* was used

It thus appeared that there was a substance in the culture fluid which was active mainly against *Myco phlei* and was not readily inactivated by trypsin, but which was lost during the extraction of alvein The antibacterial activity of the culture fluids produced by deep fermentation in Czapek-Dox broth containing high concentrations of corn steep liquor and glucose was probably due mainly to the presence of this substance

SUMMARY

An antibiotic named alvein has been extracted from the culture fluid of a strain of *Bacillus alvei* Alvein has the properties of a strongly basic polypeptide It is highly active against a variety of Gram-positive bacteria and less active against certain Gram-negative bacteria It is haemolytic

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THE ANTIBACTERIAL PROPERTIES OF SOME SPECIES OF AEROBIC SPORE-FORMING BACILLI

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In recent years some 20 apparently distinct antibiotics have been obtained from the culture fluids of various species of aerobic spore-forming bacilli. A small number of them are capable of protecting animals against experimental infections with bacteria susceptible to their action. Some have been used in man, particularly for local application on infected surfaces.

This paper records the results of a survey of the antibacterial activity of identified strains of bacilli. The object of the survey was to select strains, if possible not previously investigated, as a preliminary to more detailed investigation of their antibacterial properties.

EXPERIMENTAL

Most of the cultures were obtained from the National Collection of Type Cultures, or from the collection of Dr T. Gibson, of Edinburgh, and the identity of all strains was established or checked by the methods of Gibson (1944) and Gibson and Topping (1938). To keep the scope of the work within practicable limits the strains were examined by a restricted number of somewhat arbitrary procedures. The procedures were designed to detect those strains which secreted antibacterial substances, and to differentiate as far as possible between different substances. Strains were selected for further study if they produced large zones of inhibition in any of the tests, and if the antibiotics concerned appeared to possess interesting antibacterial ranges. It was also considered advantageous if the substances were not readily inactivated in the presence of trypsin or serum.

Detection of active strains

The production of antibacterial substances at 37° C on one solid medium and in one liquid medium was studied.

Solid medium.—The solid medium was a lemco agar containing 1 per cent peptone, 1 per cent lemco and 0·5 per cent sodium chloride, all the strains being tested on this medium by the cross-streak method. Primary streaks of bacilli were incubated for 24 hours, and the test organisms were then streaked at right angles to them. After a further 24 hours' incubation measurements were taken of any inhibitions. The following were used as test organisms:

Staph. aureus N C T C 6571, *Bact. coli*, *Ps. pyocyanea*, *S. enteritidis* Gaertner, *S. typhi*, *C. xerosis*, *C. diphtheriae* var. *gravis*, *Myco. phlei* (laboratory strain, N C T C strains 525 and 1471) and *Myco. smegmatis*

Liquid medium —Potato dextrose broth was chosen as the liquid medium since it had already been found to be suitable for the production of bacilysin by a strain of *B subtilis* and of ayfivin by a strain of *B licheniformis* (Arriagada, Abraham, Sharp, Savage, Sharp and Heatley, 1949) In making a litre of broth, 200 g of diced potatoes were steamed in 500 ml of tap water for one hour After the bulk of the debris had settled the liquor was strained through cotton-wool, the volume made up to 1 litre with tap water, and 10 g of dextrose added The bacilli were grown in layers of broth about 1 cm deep in 500 ml Erlenmeyer flasks The activity of the culture fluids was assayed daily for 12 days by the cylinder plate method (Heatley, 1944), using *Staph aureus* and *Myco phlei* as test organisms The antibacterial content of the cells was not investigated

Differentiation between the antibacterial substances produced

Some differentiation between the substances secreted on the cross streak plates was possible on the basis of the differences in antibacterial ranges

Several methods were used in attempts to differentiate between the substances secreted into the liquid medium

(*a*) Culture filtrates were sterilized by gradocol filtration, and incorporated with agar in gutters cut along the diameter of lemco agar plates After diffusion overnight at 4° C these were cross streaked with a range of bacterial test organisms, and inhibitions were measured after overnight incubation at 37° C

(*b*) Samples of culture filtrates were incubated for 6 hours, either with 1 mg per ml of commercial trypsin, or in 50 per cent horse serum, and the residual activity was then measured by the cylinder plate method

(*c*) Many of the zones of inhibition of *Staph aureus* produced by the culture fluids of *B subtilis* and *B pumilus* contained scattered colonies of resistant staphylococci (Abraham, Callow and Gilliver, 1946) Plates seeded with staphylococci resistant to one culture filtrate were used for testing other culture filtrates, in the hope that the specificity of the resistant organisms would yield information of value in grouping these antibiotics

RESULTS

Active strains

The results are summarized in Table I

In all, 165 strains of bacilli were tested, and of these, 61 strains belonged to the *Bacillus subtilis* group, comprising *B subtilis*, *B pumilus* and *B licheniformis* All the strains of this group were active against one or more of the test organisms on one or both media, only one strain failing to secrete active substances into lemco agar and five into potato dextrose broth Strains of other species of bacilli were considerably less active, only about one-third showing activity on the solid medium, and only two strains, both of *B cereus* secreting active substances into potato dextrose broth The species showing activity on lemco agar were *B brevis*, *B alvei*, *B laterosporus*, *B circulans*, *B megatherium* and *B polymyxa*

The importance of pellicle formation

The secretion of antibacterial substances into potato dextrose broth by strains of the *B subtilis* group was correlated with their ability to grow in the form of a

pellicle   All the consistently active strains formed thick pellicles on potato dextrose broth and the five inactive strains failed to form any surface growth Five strains varied from time to time both in their ability to form pellicles and to secrete antibacterial substances, and the two characteristics were closely connected   Cultures of those strains which had formed thin pellicles and secreted active substances were not appreciably more turbid when shaken than entirely submerged cultures of the same strain which were inactive

Differentiation between the antibiotics produced

Solid medium —It had been hoped that it would be possible to classify the antibiotics produced in agar into several groups by the differences in antibacterial range shown on the cross-streak plates   Upon repetition of these tests, however, it was shown that for most strains the inhibition of *Staph aureus* and of the Gram-negative test organisms was erratic, and that the only criteria sufficiently reliable for this purpose were the relative inhibition of the various strains of *Myco phlei*, and the relative inhibition of *C xerosis* and the laboratory strain of *Myco phlei*   The actual figures obtained with any strain varied from day to day, but the relative inhibition of these test organisms was unchanged   Zones of inhibition of greater width than 15 mm   were rarely recorded

Two main groups were found

Group 1 —The zone of inhibition of *Myco phlei* N C T C 1471 was greater than or equal to that of the laboratory strain, and the zone of inhibition of the N C T C 525 strain was appreciable   The zone of inhibition of *C xerosis* was also equal to or greater than that of the laboratory strain of *Myco phlei*

Group 2 —The zone of inhibition of Strain 1471 was less than that of the laboratory strain, the difference being more than 3 mm   The inhibition of Strain 525 was slight, and that of *C xerosis* was less than that of the laboratory strain, the difference being again more than 3 mm

Most of the results fell into either of these two groups, only a small number of intermediate types remaining

Among the *B subtilis* group, all the strains of *B licheniformis* exhibited the Group 1 type of antibacterial range, and the strains of *B subtilis* and *B pumilus* in general gave the Group 2 results, a few showing either Group 1 or intermediate results   The same two types of antibacterial range were found among the other species of bacilli, different strains of the same species sometimes showing different pictures

Liquid medium —The results obtained with the substances secreted in potato dextrose broth are summarized in Table I   Specific points are discussed in the following paragraphs

The active substance secreted into potato dextrose broth were of two main types   The first inhibited the growth of *Staph aureus* in the cylinder plate tests, and the second inhibited the growth of *Myco phlei*   The two types of substances were secreted independently of each other in the same culture fluids

The evidence for this distinction was as follows

(1) The culture fluids of some strains which were usually active against both test organisms were occasionally active only against one of them

(2) In some instances the peak activity against one test organism was attained before that against the other

TABLE I

Antibacterial substances in culture fluid

Unconcentrated substance active in cylinder-plate test against *Staphylococcus aureus*		Unconcentrated substance active in cylinder plate test against *Mycobacterium phlei* only	
BACILYSINS secreted by *B subtilis* and *B pumilus*	Substances secreted by *B licheniformis*	Secreted by *B licheniformis*, and a few strains of *B subtilis*	Secreted by most strains of *B subtilis* and *B pumilus*
Resistant staphylococci show cross-resistance within this group	Staphylococci resistant to bacilysins are still sensitive to these substances	Antibacterial range on gutter plates	Antibacterial range on gutter plates
		Group 1	Group 2
All inactivated by trypsin	Inactivated by trypsin / Not inacti vated by trypsin	Both groups subdivided by relative activity in the presence of trypsin or of serum	
Possibly subdivided by relative activity in presence of serum			

(3) The effect of incubation with trypsin upon the culture fluids of many strains was to remove all the inhibitory action against *Staph aureus*, while that against *Myco phlei* remained unchanged or was somewhat reduced

Of the substances inhibitory against *Staph aureus*, those secreted by strains of *B subtilis* and *B pumilus* were provisionally called bacilysins, since they were apparently indistinguishable from each other and from a substance secreted by a strain of *B subtilis*, N C T C 7197, to which this name had originally been given (Abraham, Gilliver and Florey, 1947, unpublished)   The bacilysins were inactivated by incubation with trypsin and were insoluble in organic solvents Staphylococci resistant to the bacilysin secreted by one strain of *B subtilis* were also resistant to those secreted by other strains   They were, however, still sensitive to the substances active against *Staph aureus* which were secreted by strains of *B licheniformis*   The latter substances therefore appeared to be distinct from the bacilysins

The substances active against *Myco phlei* in the cylinder plate tests appeared to be responsible for the two types of antibacterial range that occurred when culture fluids were tested by the gutter plate method   This was shown by the fact that when the bacilysin content of culture fluids was destroyed by incubation with trypsin, the inhibitions on gutter plates remained unchanged except for a reduction in the activity against *Staph aureus*   Moreover the failure to distinguish between the bacilysins by the very sensitive method of tests with resistant staphylococci showed that these substances were unlikely to exhibit differences in antibacterial range

The differentiation on the gutter plates between the substances active in cylinder plate tests against *Myco phlei* depended, as in the cross-streak plates, upon the relative inhibition by them of *C xerosis* and *Myco phlei*   In Group 1 the inhibition of *C xerosis* was greater than or equal to that of *Myco phlei* (laboratory strain), and in Group 2 the inhibition of *Myco phlei* was considerably greater than that of *C xerosis*, which frequently was not inhibited at all

The Group 1 picture was characteristic of the substances secreted by *B lichcni-formis*, whereas those secreted by *B subtilis* and *B pumilus* usually gave the Group 2 picture   A few strains of *B subtilis* showed the Group 1 range   There were no intermediate types of result

The substances secreted by *B cereus* were inactive against *Staph aureus* when tested by the cylinder-plate method, but remained unclassified by the gutter plate method since they were apparently inactivated by gradocol filtration The activity in these culture fluids was increased, however, by incubation with trypsin, and this distinguished them from the culture fluids of all other species

TABLE II

Species	Number tested	Active in potato dextrose broth	Activity on gutter plates Number of strains Group		Activity on cross streak plates Number of strains Group			
			1	2	1	2	Inter mediate	Inactive
B subtilis	36	35	8	27	1	24	9	1
B lichcniformis	18	18	18	—	17	—	1	—
B pumilus	7	3	—	3	1	1	4	1
B cercus	16	2	—	—	1	7	3	5
B brevis	13	—	—	—	5	—	5	3
B laterosporus	5	—	—	—	2	1	1	1
B alvci	9	—	—	—	6	1	—	2
B circulans	11	—	—	—	1	2	—	8
B megatherium	13	—	—	—	—	1	—	12
B polymyxa	2	—	—	—	—	1	—	1
B fusiformis	10	—	—	—	—	—	—	10
B carotarum	9	—	—	—	—	—	—	9
B mycoides	8	—	—	—	—	—	—	8
B maccrans	6	—	—	—	—	—	—	6
B coagulans	3	—	—	—	—	—	—	3
B firmus	1	—	—	—	—	—	—	1

Selection of strains for further investigation

The following strains were selected for further study
    B subtilis A 14
    B alvci Gibson strains 642, 949, 1067
    B laterosporus Gibson strains 1066, 1080
    B brevis N C T C 5402, Gibson 442
    B circulans N C T C 6351
    B polymyxa N C T C 4747

The strain of *B subtilis* was selected on the grounds of its high activity in potato dextrose broth   Although the zones of inhibition produced by the culture fluids of most strains of *B subtilis* rarely reached 30 mm in diameter, the culture fluid of this strain contained substances which caused zones of inhibition of 30–35 mm in diameter and 22–25 mm in diameter respectively on plates seeded with *Myco phlci* and *Staph aureus*   The activity against *Myco phlei* was only slightly reduced by incubation either with trypsin or with serum   The antibacterial substances secreted by this strain have been investigated in some detail (Newton, to be published)

The other strains showed on cross streak plates appreciable inhibition of the Gram-negative bacteria in addition to a high activity against the Gram-positive

bacteria, and tests on blood agar showed that these effects were not due to the presence of hydrogen peroxide

Investigations have been made of the substances secreted by one strain each of *B laterosporus* and *B alvei* (Barnes, 1949 , Gilhver, Holmes and Abraham, 1949)

<center>•</center>

<center>DISCUSSION</center>

It is recognized that the restriction of the number of tests employed imposed certain limitations upon the survey The methods of assay, for example detected only those substances that were diffusible in agar, and it is also possible that different results would have been obtained by incubating cultures at several different temperatures and in several different media Lemco agar was a more successful choice than potato dextrose broth in the latter respect, since many of the bacilli other than the *B subtilis* group secreted active substances when grown on it It is unlikely, however, that any one liquid medium would have been useful for all the species, and the use of potato dextrose broth appeared to be justified at least in the case of the *B subtilis* group The choice of test organisms might also be said to have been somewhat restricted Nevertheless it is thought likely that a fairly good idea of the antibacterial possibilities of the bacilli was obtained

It seems clear that the substances secreted on the same medium by all strains of *B licheniformis* were similar to each other in antibacterial range, and distinct from the corresponding substances secreted by most strains of *B subtilis* and *B pumilus* Further distinctions between these groups of substances appeared to necessitate the use of methods outside the scope of this survey The occurrence of intermediate types of result on cross streak plates, for example, suggested that there was a range of substances being secreted, those at the two extremes falling into the arbitrarily defined Groups 1 and 2 The groups themselves each consisted therefore of a collection of substances with a broadly similar antibacterial range, but not necessarily with any other property in common An experiment in which both primary streaks and test organisms were bacilli with the Group 1 antibacterial range demonstrated clear-cut differences of range within the group , but an extension of this experiment was unfortunately impracticable

The demonstration of the importance of pellicle formation in the production of antibiotics by the *B subtilis* group is of some interest One effect of the formation of a surface pellicle is that the improved aëration permits of an increased growth of the organism, and the secretion of detectable amounts of antibacterial substances under these conditions may be a direct consequence of this Observations made with submerged cultures of *B subtilis* strain A 14 and with surface cultures of *B alvei* G 642 tend to support this view The secretion of antibacterial substances by these bacilli was increased or modified when growth was stimulated, either by more efficient aëration methods in the first instance, or by the support of a surface growth of the latter organism by mechanical means (Newton, unpublished , Gilliver *et al* , 1949)

The phenomena exhibited by the five variable strains of *B subtilis* and *B pumilus* could not be explained in this manner Nor in the case of the strain of *B alvei* referred to above, was aëration and consequent increased growth the decisive factor, since media were developed on which thick pellicles were formed

but into which no antibiotic was secreted   In the former case the secretion of detectable amounts of antibiotics which followed the establishment of a surface growth seemed to result from an increased or modified metabolic activity of the cells already present, rather than from a continued activity of an increased number of cells   In the case of *B alvei*, factors such as carbon and nitrogen sources, growth factor requirements, temperature and hydrogen ion concentration were probably playing a part in limiting antibacterial activity

The exact relationship between the substances demonstrated in this survey and those already described in the literature is not certain   The group of substances secreted in potato dextrose broth and inhibitory towards *Myco phlei* probably comprises many different types, those secreted by the two strains of *B cereus* presenting certain features of interest   Of the strains selected as the result of the cross streak tests, the two so far investigated, *B alvei* and *B laterosporus*, have been found to secrete in certain liquid media substances distinct from those already described (Gilliver *et al*, 1949, Barnes, 1949) While it is by no means certain that these organisms secreted the same substances into both the liquid medium and the solid medium, it would appear likely that an investigation of other strains selected by the cross-streak method would be worth while

SUMMARY

One hundred and sixty-five strains of aërobic spore-forming bacilli were tested for their ability to secrete antibacterial substances into lemco agar and into potato dextrose broth   All the strains of the *B subtilis* group were found to be active in one or both media   Only one-third of the strains of other species showed any activity, and this was confined to lemco agar   It was found possible to group the substances produced on the basis of their antibacterial ranges, and by the effect of trypsin and serum upon them   Certain strains have been selected for more detailed investigation

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SEROLOGICAL DIAGNOSIS IN MUMPS

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HABEL'S discovery in 1945 that mumps virus can be cultivated in chick embryos (Habel, 1945) has made laboratory work with mumps possible on a larger scale   It also makes serological diagnosis possible   The serological diagnosis is of special interest in clinically doubtful cases and in patients with encephalitis of unknown etiology, a certain number of which are due to mumps virus (Kane and Enders, 1945)

The purpose of this paper is to investigate the reliability of complement fixation and haemagglutination inhibition tests as diagnostic methods in mumps

MATERIAL

Sera were collected from 60 patients admitted for mumps in the hospital for infectious diseases in Stockholm   All patients had mumps with classical symptoms   In the material neither doubtful cases nor cases with encephalitis without symptoms from the salivary glands are included   Sera were taken as early as possible during the illness and subsequently every tenth day

The material is not representative of mumps in general   It is selected according to the indications for hospitalization   The rate of complications, especially encephalitis and orchitis, is higher than usual in mumps

Sera from 51 adults, who had not had mumps during the last two years, were used as a control material

The strain used was kindly supplied by Dr G Henle in Philadelphia   In this paper it is referred to as the A-strain   This strain was used throughout for the experiments unless otherwise stated   For cross-inhibition tests another three strains were used   Two of them, the WAL and STO strains, were forwarded by Miss Patricia Lind in Melbourne   The third one, the V strain, was isolated in this laboratory during an epidemic of mumps in 1947–48   The sera were collected during the same epidemic

The V strain was isolated in the amniotic cavity   Saliva was taken on the second day of illness, was mixed with 1500 units of penicillin and 1000 µg of streptomycin per ml   and injected in an amount of 0 2 ml   into the amniotic cavity   The saliva had been kept at − 70° C   for about a month before being tested   The strain showed agglutination of guinea-pig and chicken red cells from the first passage   In spite of a great number of allantoic passages it could not be adapted to the allantoic cavity

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METHODS

Cultivation of virus

The A strain was maintained through allantoic passages in eggs 7 days old, using infected allantoic fluid in a dilution of 10^{-2} as inoculum The allantoic fluid was harvested after 6 days' incubation of the eggs at 35° C Under these conditions the best yield of virus was obtained The strain grew irregularly in the allantoic cavity, the logarithmic haemagglutination titres ranging from not measurable to 3 01 and seldom higher Thus it was necessary to test the harvested fluid from each egg for the presence of virus

Complement-fixation test

To a series of 0 2 ml twofold serum dilutions from 1/4 to 1/512 0 2 ml of antigen and 0 2 ml of complement were added The mixture was incubated in a waterbath at 37° C for 45 minutes After addition of 0 5 ml of sensitized sheep red cells the tubes were left in the waterbath for another 30 minutes and then placed in a cold room at 4° C overnight The test was read the following morning

Antigen – An infected allantoic fluid was used, which had been diluted 1/2 to 1/8 according to a preceding antigen titration against a standard serum It was kept frozen at −16° C until used Merthiolate 1/10,000 was used as preservative

Complement —Guinea-pig complement was titrated in the presence of the same amount of antigen as used in the test The amount of complement in the last tube with complete haemolysis was taken as a unit In the test 1 5 units were used The complement was stored at −16° C and discarded if more than one week old

Sensitized sheep red cells —Red cells were washed three times and used in a dilution of 0 75 per cent Amboceptor was added to the red cell suspension in an amount corresponding to 4 units per tube The mixture of red cells and amboceptor was allowed to stand for 15 minutes before being used

Serum —All sera were inactivated at 56° C for 30 minutes Sera from the same patient were titrated simultaneously with the same antigen and the same complement dilutions They were kept frozen at −16° C

Controls —(1) One tube containing all reagents except antigen (2) One tube in which normal allantoic fluid was substituted for antigen (3) Control with negative serum (4) Control with positive serum

The titres are expressed throughout as the negative log of the initial serum dilution that is the serum dilution before antigen, complement and sheep red cells have been added The calculated 50 per cent haemolysis limit was taken as endpoint of the complement-fixing capacity

The agglutination titres of the virus suspensions were determined according to Salk (1944)

Haemagglutination inhibition test

Salk s modification of Hirst s technique was used throughout (Salk, 1944) The sera were thus diluted in 0 5 ml of 0 25 per cent chicken red cell suspension and titrated with the same amount of a virus suspension containing 4 units of virus, the amount of virus causing a $\pm$ reaction in haemagglutination titration

taken as a unit   The tubes were left at room temperature for 1½ hours and then placed at 4° C overnight   The test was read the following morning by observing the bottom patterns

In 1948 Hirst reported that the normal inhibitor of serum could be destroyed by treatment of the serum with sodium periodate   This report indicated a way to investigate whether the lack of correlation between haemagglutination inhibition and complement-fixation titres (see below) might depend upon the disturbing effect of the normal inhibitor   In investigating this possibility, treatment with periodate was performed in the following way   0 2 ml of serum was mixed with 0 2 ml of 0 05 M NaIO$_4$ and was allowed to stand for 2 hours   The excess of periodate was then neutralized by the addition of 0 8 ml of 5 per cent glucose

Each serum was titrated simultaneously with and without treatment with periodate

Sera from the same patient were titrated at the same time   They were inactivated at 56° C for 30 minutes

The titres assigned to the sera are expressed as the negative log of the final serum dilution after addition of all reagents   The tube with a $\pm$ reaction, or if there were more than one, the first one of them, was taken as endpoint of the inhibiting capacity   If there was no tube with $\pm$ reaction the endpoint was calculated through interpolation between the last tube with no agglutination and the first tube with total agglutination

The reading was sometimes considerably hampered by the fact that the limit between agglutination and inhibition was not sharp enough   The endpoint of some sera was repeatedly so diffuse that no satisfactory titre could be determined and the serum had to be excluded from the investigation

EXPERIMENTAL

Reproducibility of complement-fixation, haemagglutination and haemagglutination inhibition titres

In order to assess the reproducibility of the titres a number of titrations with each of the methods were carried out   In the haemagglutination inhibition and complement-fixation titrations a known positive serum with a high complement-fixation and inhibition titre was used   Each method was tested in the course of one day and with the same dilutions of the participating reagents throughout the experiment   .

Complement-fixation test —The greatest difference between two titres of a total of 50 was 0 24   The standard deviation (σ) was 0 056, the standard deviation of σ 0 0056 and thus $3\sqrt{2}\,\sigma = 0\ 24$ Accordingly a difference corresponding to one tube, that is 0 30, may be considered significant

The result is presented in Table I

Haemagglutination titration —The precision of haemagglutination titres was studied by 59 haemagglutination titrations of the same virus suspension   The standard deviation was found to be 0 11 the standard deviation of σ 0 0101 and $3\sqrt{2}\,\sigma$ 0 47 (Table I)   The greatest difference between two titres was 0 38

Haemagglutination inhibition test —40 titrations of the same serum were carried out   The greatest difference between two titres was 0 45 and all except

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two lay within the range 3 16 and 3 46   The standard deviation was 0 120, the standard deviation of σ0 0134 and $3\sqrt{2}\,\sigma = 0\,51$ With the same level of significance as for the complement-fixation test a difference corresponding to 2 tubes, that is 0 60, may be considered significant   The result is presented in Table I

TABLE I —*Deviations and Standard Deviation of Complement-fixation, Haemagglutination and Haemagglutination Inhibition Test*

	Number of titrations	Mean of titres	Deviation from mean		Standard deviation σ	Standard deviation of $\sigma\left(\dfrac{\sigma}{\sqrt{2n}}\right)$	$3\sqrt{2}\,\sigma$
			Greatest	Least.			
Complement fixation test	50	1 71	0 14	0 01	0 056	0 0056	0 24
Haemagglutination test	59	2 57	0 22	0 01	0 11	0 0101	0 47
Haemagglutination inhibition test	40	3 31	0 30	0 15	0 120	0 0134	0 51

If haemagglutination inhibition titres are determined at different times there are two more variable factors introduced into the test, namely, different virus and different red cell suspensions

The effect of variation of the amount of virus was studied by titration of a known positive serum with a high inhibition titre with 4–512 units of virus The result is presented in Fig 1

It is evident that there exists a linear relationship between log amount of virus and log inhibition titre   A fourfold increase of the amount of virus corresponds to a twofold decrease of serum titre   The inhibition titres are thus influenced by variation of the amount of virus to a comparatively slight extent A similar relation between the amount of influenza virus and the corresponding mouse neutralization titre was earlier found by Horsfall (1939)

The haemagglutination titres may differ by 0 47 (see above) when the titrations are made on the same day and with the same dilutions of the reagents This variation of the virus concentrations of the dilutions used for antibody titrations may cause a difference of 0 24 of inhibition titres, thus increasing the variations of inhibition titres to 0 75   A still greater difference of inhibition titres, 0 90, was found in duplicate titrations of 26 sera on different occasions This difference might be explained by the use of different red cell suspensions

In investigating the effect of variation of red cell suspensions it was found that titrations with red cells from different hens gave surprisingly great differences of titres   Differences of 1 5 and 1 8 were not uncommon   This is in agreement with the report of Anderson, Burnet and Stone (1946) that non-specific inhibitory titres showed a great variation when they were determined with red cells from different fowls, as well as Svedmyr's finding that titration of the inhibiting effect of normal allantoic fluid gave different results when red cells from different fowls were used (Svedmyr, personal communication)

The greatest variation of the means of the haemagglutination inhibition titres of 22 sera, tested with 4 different kinds of red cells, was 1 05 (Table II) Eight were acute phase and 14 convalescent sera   There was no certain difference between acute phase and convalescent sera concerning the influence of different

red cells on the inhibition titres (Table II)   The maximal difference of titre values of the same serum among these 22 sera was 2 10 (7 tubes)

TABLE II —*Means of Haemagglutination Inhibition Titres of Acute Phase and Convalescent Sera not Treated with Periodate and Titrated with Red Cells from 4 Fowls*

	Fowl number			
	1	2	3	4
Acute phase sera (8) .	1 94	2 00	2 95	2 86
Convalescent sera (14)	2 00	2 10	2 95	3 13
Total (22)	1 95	2 07	2 96	3 00

After treatment with periodate, 14 sera, 9 of which were convalescent and the others acute phase sera, were titrated with red cells from 4 fowls   The greatest difference of titre of the same serum was 1 50 (5 tubes)   The mean of the titres,
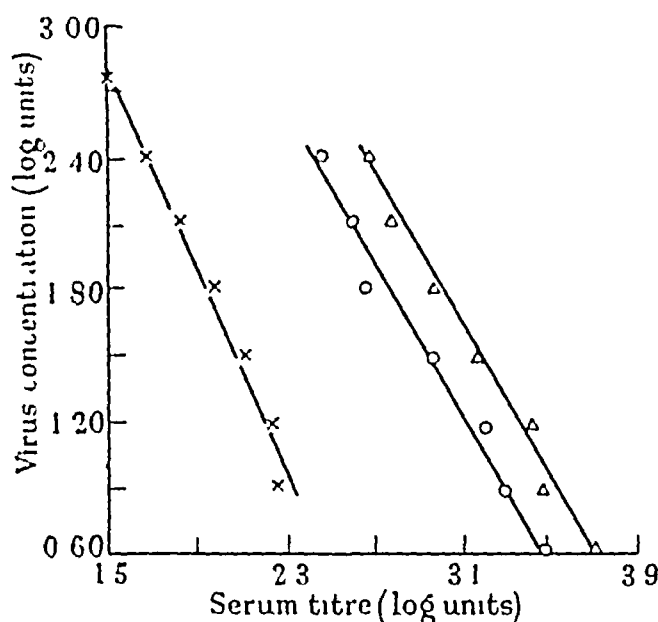
Fig 1 —Correlation between haemagglutination inhibition titre of serum and virus concentration
X   Serum no 175, no periodate
△   „   „   180, „   „
O   „   „   181, periodate

determined with red cells from the same fowl, was calculated   These calculated means varied from 1 59 to 2 17 (Table III)   The corresponding figures for acute phase and convalescent sera were respectively 1 41 to 1 95 and 1 84 to 2 43 (Table III)   Thus the variations of the titres of convalescent and acute phase sera were of about the same magnitude

TABLE III —*Means of Haemagglutination Inhibition Titres of Acute Phase and Convalescent Sera, Treated with Periodate and Titrated with Red Cells from Different Fowls*

	Fowl number			
	1	2	3	4
Acute phase sera (5)	1 41	1 38	1 95	1 87
Convalescent sera (9)	1 84	1 82	2 43	2 34
Total (14)	1 70	1 59	2 14	2 17

In general the differences were smaller when the sera were treated with periodate before being titrated   Nevertheless, they were great enough to cause misinterpretations, if titres determined at different times were compared, and they were easily demonstrable both in acute phase and in convalescent sera

Each virus suspension which was used for antibody titrations was titrated with the 4 different kinds of red cells, used for inhibition titrations   The variations lay within the distribution of haemagglutination titres, calculated above

Control material

Complement-fixation test —None of the 51 sera contained enough antibodies to cause complete fixation in a dilution of 1 4   Six of them showed partial fixation The remaining 45 sera showed no complement-fixing capacity under the conditions of the experiment

Haemagglutination inhibition test —The control sera inhibited in fairly high dilution the haemagglutination by mumps virus   As seen from Table IV, 32 of a total of 51 had an inhibition titre of 2 71 or more

TABLE IV —*Haemagglutination Inhibition Titres of Control Sera not Treated with Periodate*

	Number of cases								
	0	1	6	10	1	25	1	7	0
Titre in log units	<1 81	1 81	2 11	2 41	2 56	2 71	2 86	3 01	>3 01

Treatment of the sera with periodate strongly reduced the inhibition titres No titre exceeded 2 28 , 10 sera had a titre of 1 68 or more and 30 titres were lower than 1 38 (Table V)

TABLE V —*Haemagglutination Inhibition Titres of Control Sera Treated with Periodate*

	Number of cases							
	30	7	3	6	1	2	1	0
Titre in log units	<1 38	1 38	1 53	1 68	1 83	1 98	2 28	>2 28

Thus the control material showed a divergence between complement-fixation and inhibition titres which—as will be seen later—exists also between complement-fixation and inhibition titres of convalescent sera

Comparative titrations of acute phase and convalescent sera

The material consisted of 60 patients with clinical symptoms of mumps   In some of the cases sera were not obtainable early enough in the acute stage or late enough during convalescence   They are, nevertheless, presented in the table

For every serum three titre values are presented, viz complement-fixation titre and haemagglutination inhibition titre, determined with and without periodate

Complement-fixation —A significant rise of complement-fixing antibodies occurred in 47 patients (Table VI)   In two cases no antibodies could be

demonstrated with the technique used, although sera were available up to two months after the onset of illness In the remaining 11 cases a sufficient number of blood specimens could not be obtained A patient was not considered as negative, unless negative specimens were obtainable during acute stage and subsequently up to 40 days after the onset of illness

Haemagglutination inhibition test —Titration of sera not treated with periodate revealed a significant rise of antibodies in only 11 cases After treatment of the sera with periodate a significant rise could be demonstrated in 27 patients (Table VI)

Summing up the results, 80 per cent of the cases could be diagnosed with the aid of complement-fixation technique, and 18 per cent with the haemagglutination inhibition test Treatment of the sera with periodate augmented the rate of positive cases in inhibition titrations to 45 per cent (Table VI)

The above data provide no absolute values of the reliability of the three serological methods, as in some cases a sufficient number of blood specimens was not obtainable However, they may be used for comparison of the reliability of the methods

TABLE VI —*Titrations of Sera From 60 Mumps Patients*

	Complement fixation test	Haemagglutination Inhibition test	Haemagglutination inhibition test Periodate
Number of positive cases	17	11	27
Per cent positive cases	80	18	45

If only those cases are considered in which a significant rise of antibodies could be demonstrated with one of the tests, a comparison between the three methods gives the following result Complement-fixation test 94 per cent positive cases, haemagglutination inhibition test 26 per cent, and haemagglutination inhibition test, after treatment of the sera with periodate, 57 per cent

This result indicates a great divergence between complement-fixation and inhibition titres of the whole material

It is illustrated in Fig 2 and 3

Fig 2 illustrates the differences of the rise of the titres In this figure the mean of the individual increase of the respective titres, from the acute phase to 10 to 20, and 20 to 30 days after onset of illness, is reproduced It can be seen that the average increase of haemagglutination inhibition titres was about 0 60 (2 tubes), when the titres were determined after treatment of the sera with periodate, but only 0 30 (1 tube) when they were determined without treatment with periodate The average rise of complement-fixation titres was 1 5 (5 tubes)

From Fig 3, in which the number of cases is plotted against the rise of titres, it is evident that the treatment with periodate brings about a closer correlation between complement-fixation and haemagglutination inhibition titres On the other hand, it is also evident that the treatment with periodate does not make the divergence between the two methods disappear

In many cases there was a significant rise of complement-fixation titre, but no rise of the inhibition titre Two cases showed a significant rise of haemagglutination inhibition, but no rise of complement-fixation titre and in many cases the rise of the respective titres did not appear simultaneously

Cross-inhibition titrations with 4 different strains

The lack of correlation between clinical findings and the haemagglutination inhibition titres might be explained by differences between the strain used for titrations and the strain which caused the actual outbreak of mumps, especially
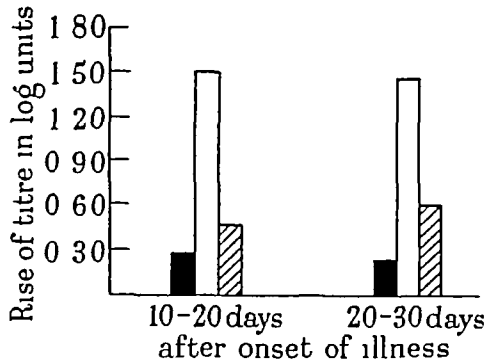
FIG 2 —Average increase in log units of complement fixation and haemagglutination titres in 60 mumps patients

■ Haemagglutination inhibition test
□ Complement fixation test
≣ Haemagglutination inhibition test   Periodate

FIG 3 —Distribution of the observed maximal increase of complement fixation and haemagglutination inhibition titres   Material comprises 39 patients observed 10–20 days, and 20 patients observed 20–30 days after onset of illness
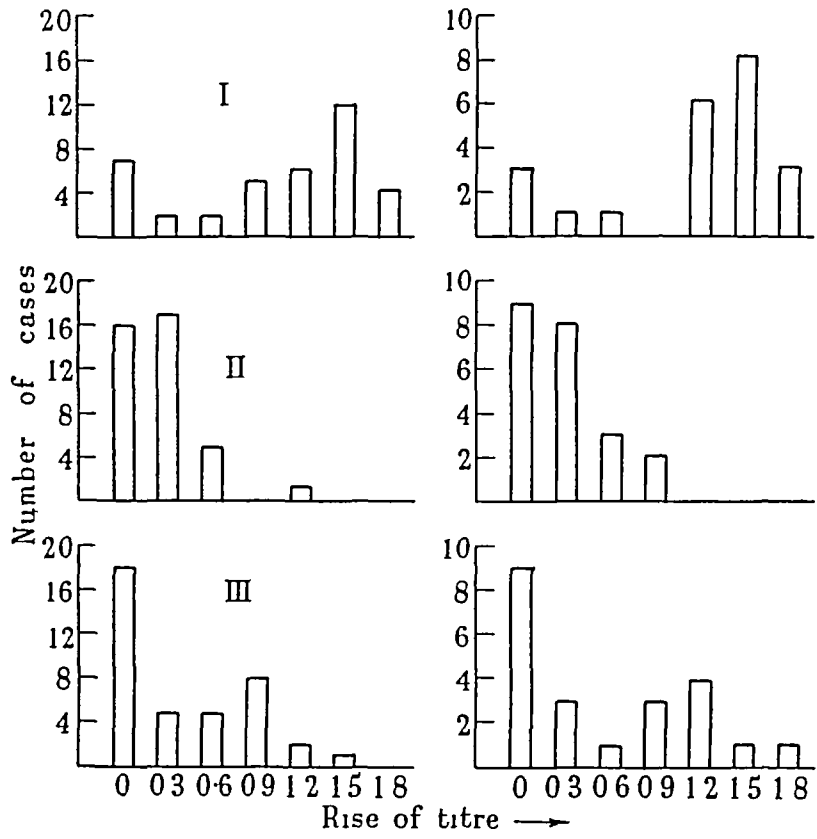
I   Complement fixation test
II   Haemagglutination inhibition test
III Haemagglutination inhibition test   Periodate

as the strain used throughout this investigation was isolated in the U S A , and the sera were collected from patients living in Sweden   Considering this possibility a number of cross-inhibition tests with 4 different strains were carried out Only the A strain being adapted to the allantoic cavity, all the titrations used for comparison of different strains were performed with infected amniotic fluid

Eighteen convalescent sera were titrated   The difference of the titres of these sera did not exceed 0 60 if they were not treated with periodate , this is within the normal variation of titres determined with different virus suspensions   None of the strains showed any tendency towards constantly higher or lower titres than the others

Titration of the same sera after treatment with periodate gave a somewhat different result   Twelve of 18 sera gave lower titres with the A strain than with the others (Table VII)   Whether this is really due to strain differences is not possible to decide

TABLE VII —*Cross-inhibition Test with 4 Strains against Human Convalescent Sera, Treated with Periodate*

Serum	Virus strain			
	A	V	WAL	STO
1	1 38	1 38	1 38	1 38
2	2 58	2 58	2 58	2 58
3	1 38	2 89	2 58	2 58
4	2 28	3 19	2 89	2 89
5	1 98	3 19	2 89	2 89
6	1 68	1 98	1 98	1 98
7	1 68	2 58	2 58	2 58
8	2 58	2 58	2 28	2 28
9	2 58	3 19	3 19	2 89
10	2 89	2 28	2 58	2 28
11	2 28	2 58	2 58	2 58
12	3 49	3 19	2 58	3 19
13	1 68	1 68	1 68	1 68
14	1 98	3 19	3 49	2 89
15	1 38	2 28	2 58	1 68
16	1 38	2 58	2 58	1 98
17	2 58	3 19	2 89	3 19
18	1 68	2 89	2 58	2 28

Titration with homologous strain.

Titration of a number of sera against the V strain, which was isolated from a patient out of the outbreak of mumps, during which the sera were collected, showed the same divergence between complement-fixation and haemagglutination inhibition titres   Of 19 patients, 1 showed a rise of inhibition titre of 0 90, and 6 a rise of 0 60   Treatment of the sera with periodate revealed a significant rise of inhibition titres in 3 patients   In all except 1, complement-fixing antibodies could be demonstrated in significant amounts

DISCUSSION

On account of the high haemagglutination inhibition titre values of the control material it is evident that no conclusions as to infection with mumps virus can be drawn from a single haemagglutination inhibition titre   The control material is too limited to allow any conclusions whether the diagnosis can be established with the aid of a single complement-fixation titre

The haemagglutination inhibition reaction is extensively used, both for experimental and practical purposes, especially in work with influenza virus In spite of this fact, the experience and the knowledge of the reaction is still too limited to allow a definite judgment about its specificity and its value

The data presented indicate that the haemagglutination inhibition reaction has a very limited value in serological work with mumps, both in its original and in its modified form   Evidently, it failed to reflect the actual antibody content of the sera in the material presented

The great differences of haemagglutination inhibition titres which are determined with red cells from different fowls, emphasize the fact that inhibition titres should not be compared unless they are determined with the same red cell suspension   The causes of these great variations are still obscure   They do not occur only in titrations of " normal " sera, but also in sera containing specific inhibiting antibodies, and they do not disappear after treatment of the serum with periodate   If Hirst's hypothesis, that sodium periodate destroys the normal inhibitor of serum, is correct, the differences of titres should, thus not at least entirely, be depending upon variations of the action of the normal inhibitor   The factor responsible for the differences is probably to be found in the serum, not in the virus or red cells, as the haemagglutination by mumps virus was not influenced by different red cells   A possible explanation of the phenomenon is that the sera contained two or more inhibiting factors, one of which was destroyed by periodate

The divergence between the complement-fixation and the haemagglutination inhibition titres could possibly be explained by the hypothesis that the sera tested contain antibodies which do not fix complement   However the lack of correlation between clinical findings and inhibition titres seems to exclude such a possiblity

It is not very likely that strain differences are responsible for the divergence of the titres   Comparative titrations with 4 different strains did not indicate any certain strain differences, and titrations of some of the sera with the virus strain causing the actual outbreak of the disease did not alter the results

Reports of experiences with the haemagglutination inhibition test in mumps are still very scarce, and there is no investigation (on mumps) with which the results could be compared   However, Hoyle and Fairbrother, in 1947, reported that the complement-fixation test gave more clear cut results, and higher incidence of positive reactions in influenza, than the haemagglutination inhibition reaction   Stuart-Harris and Miller (1947) interpreted some peculiar and contradicting results with the inhibition test in influenza as depending upon the fact that the strains were newly isolated   It is possible that the inhibition reaction works better in titrations with influenza virus   In any case, as far as mumps is concerned, the reaction needs further investigation as to its specificity before it can be applied for practical serological work

The complement fixation reaction yielded results which agreed with the clinical findings in all except two cases   This is in agreement with other reports of the test in mumps (Enders, Cohen and Kane, 1945)

SUMMARY

In a material consisting of 60 mumps patients the complement-fixation test yielded results in agreement with clinical findings in all except two cases

In 49 mumps patients a significant rise of antibodies could be demonstrated in 94 per cent with the aid of complement-fixation technique, in 26 per cent with haemagglutination inhibition test, and in 57 per cent with haemagglutination inhibition test after treatment of the sera with periodate   Haemagglutination inhibition titrations of the same serum with red cells from different hens gave variations of the logarithmic titres up to 2 10 (7 tubes)

Cross-inhibition tests with 4 strains, one of which was isolated during the actual outbreak of the disease, did not reveal any certain strain differences

It is concluded that the haemagglutination inhibition test failed to reflect the actual antibody content of the sera of the material presented

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AN ELECTROPHORETIC STUDY OF THE COMPONENTS OF THE SERUM PROTEINS IN CIRRHOSIS OF THE LIVER

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THE fundamental importance of the liver in protein metabolism has been firmly established by the experimental studies of Mann and his collaborators (Bollman and Mann, 1936), coupled with extensive studies of Myers and Keefer (1935), and Tumen and Bockhus (1937) on the circulating proteins in patients suffering from long standing damage to the liver parenchyma

In 1929, Abrami and Wallich in France, and Salvesen in Scandinavia, using the salting out techniques then available, demonstrated the marked alteration in the ratio of albumin to globulin in the circulating proteins of patients, associated with chronic liver damage   In the years that followed, their principal findings were confirmed by other workers

The utilization of the improved technique for electrophoresis, introduced by Tiselius (1939), offered a convenient means of making a more exact study of the changes in the circulating protein pattern associated with chronic progressive liver damage   Leutscher (1940) reported isolated analyses on two cases and followed this (1941) by single analyses of two additional cases   Further analyses

were published by Gray and Barron in 1943, and by Thorn, Armstrong and Davenport in 1946

Incidental analyses of sera from patients suffering from chronic liver damage are recorded by Kabat, Hanger, Moore and Landow (1943) in a study of the cephalin flocculation test, and by Malmross and Blix (1946) in a study of the erythrocyte sedimentation rate

CLINICAL MATERIAL

An examination of available data suggested that it would be useful to investigate sera from patients suffering from chronic progressive liver damage in whom there was no other primary condition likely to effect the analysis   For this reason, cases of carcinoma of the bile ducts, carcinoma of the liver, haemachromatosis, and other primary conditions with which degenerative changes of the liver may be associated, were excluded   All patients have been followed for at least two years or until death   Diagnoses have been substantiated in five instances by complete autopsy and in two by laparotomy   Not less than two analyses have been carried out in every case   Certain ancillary biochemical investigations performed simultaneously are listed in Table I   Patients were afebrile when serum was collected for analysis

METHODS

Blood was drawn from patients while fasting and the serum dialyzed for 72 hours against three changes of phosphate buffer of ionic strength 0 2 and pH 8 0   The analyses were carried out in the Tiselius apparatus at 0 8° C The concentration of protein in the solution actually submitted to electrophoresis was adjusted to 2 0 per cent $\pm$ 0 2 per cent in all analyses

Cholesterol estimations were carried out by a modification of the method of Schoenheimer and Sperry (1934)

Normal levels by the method used tallied closely with the careful analyses carried out by Gardner and Gainsborough (1930)   The thymol turbidity tests were carried out by the technique of Maclagan (1944a)   Alkaline phosphatase levels are given in King units, the technique followed being that of King and Armstrong (1934)   The bilirubin levels were estimated by the method of King, Haselwood and DeLory (1937)

RESULTS

The results are shown in Table I   Of the observed patients, No 2, 5 and 9 gave histories of attacks of infective hepatitis 4 years, 3 years and 6 years previously, others in the series gave equivocal accounts of ill health which might have been attacks of infective hepatitis   It was considered wiser to disregard these   Total protein levels vary from 4 0 to 8 5 g per 100 ml serum , analyses 2b and 6b, both obtained on the day of death, show totals of 4 0 and 7 6 g per 100 ml serum respectively   It is evident that the total protein levels may bear little relation to the severity of the disease   In every case there was a gross decrease in albumin level but it was noticeable that the degree of ascites and general oedema did not correspond regularly with the albumin level   Thus, in spite of an albumin level falling to 2 0 g per 100 ml , Case 9 showed no ascites nor was oedema demonstrable even when the patient was ambulant   Only

TABLE I

No	Sex	Age	Total protein g/100 ml	Albumin g/100 ml	Globulin g %				Bilirubin mg %
					α_1	α_2	β	γ	
1	Male	55	7 9	2 6	0 6	0 7	1 2	2 8	4 0
			7 7	2 7	0 4	0 7	1 1	2 8	3 0
2	,,	46	4 8	1 6	0 45	0 4	0 9	1 45	2 5
			4 0	1 1	0 4	0 4	0 8	1 3	3 0
3	,,	56	6 6	2 05	0 6	0 6	1 2	2 0	2 0
			6 0	2 0	0 5	0 5	1 1	1 9	3 0
4	Female	45	7 3	2 5	0 75		1 0	3 0	1 75
			8 5	2 9	0 8		1 5	3 5	1 0
5	,,	55	6 4	2 5	0 6		0 8	2 4	1 5
			5 6	2 3	0 2	0 3	0 7	2 1	1 5
6	,,	56	8 0	1 5	0 25	0 4	1 05	4 8	3 0
			7 6	1 4	0 2	0 3	0 9	4 7	
7	Male	42	7 0	2 2	0 90		1 2	2 6	1 5
			8 0	2 4	0 90		1 6	3 0	0 4
8	Female	65	4 7	1 8	0 35		0 9	1 6	1 0
			4 8	1 9	0 4		0 7	1 8	1 5
9	,,	64	8 2	2 0	0 3	0 3	0 8	4 8	2 0
			8 0	2 0	0 2	0 25	1 1	4 3	0 9
Normal			7 0	4 0	0 3	0 4	0 7	0 7	0
			—7 5	—4 5	—0 5	—0 6	—1 2	—1 0	—0 75

No	Thymol turbidity units	Alkaline Phosphatase units	Total Cholesterol mg/100 ml	Approximate Duration from original diagnosis	Ascites
1	8			2 years	0
				2 years and 1 month	0
2	8		190	2 years	+
	8			2 years and 14 days (Died day of second analysis)	+
3			200	3 years	+
	8	·		3 years and 2 months (Died 3 months later)	+
4	8	20		2 years	slight oedema
	10	24	170	2 years 6 weeks	slight oedema
5	14	8	180	1 year	++
	14	6		1 year and 2 months (Died 3 weeks later)	++
6	9	16	190	1 year	+
	10			1 year and 7 days (Died day of second analysis)	+
7	12	16		1 year and 10 months	0
	8	11	170	2 years	0
8	8	12	155	Probably at least 5 years	++
	8		165	2 months later	++
9	14	6	170	1 year and 6 months	0
	16	9	180	1 year and 7 months	0
Normal	0	3	150		
	—4	—12	—220		

The total protein concentration is calculated from Kjeldahl estimation. The albumin and globulin concentrations are calculated from electrophoretic analysis. The low values for α- and β globulins justify this procedure

two analyses out of 18 showed β-globulin concentrations above the normal limits though in each case the total cholesterol in the serum was normal   For simplicity the σ-globulins have been considered together   In 18 analyses, σ-globulin was above normal limits in two, and below normal limits in 8, of the remaining eight, five fell below the mean of normal

The circulating bilirubin levels were for the most part above normal levels though not of the degree usually associated with obstruction   The thymol turbidity levels were well above normal in every case   They showed little tendency to fluctuation, nor did their relative levels bear close relation to the patients' general condition   Thus Patient 9 with a thymol turbidity of 16 units was anxious to return home, while Patient 2 died with a thymol level of 8 units

In those patients in whom observations on the level of alkaline phosphatase in the serum were carried out no gross increases were observed   The highest recorded was 24 in Patient 4   Maclagan (1944b) records that 6 out of 14 cases of cirrhosis studied by him had alkaline phosphatase levels between 14 and 24   The levels of serum cholesterol were all within normal limits

DISCUSSION

The precautions taken in selecting cases for study in the series have already been stressed   Previous workers have included in their studies patients suffering from carcinoma of the bile duct, cholelithiasis with stone in the common duct, and xanthomatosis

✓   'The lack of correlation between the total protein levels and clinical severity has already been stressed, as has the lack of correlation between the levels of circulating albumin and the extent of fluid accumulation   The total circulating albumin was decreased in every analysis and is presumably the consequence of the failure of the damaged liver parenchyma to elaborate albumin

The work of Blix in 1941 indicated that a considerable proportion of the lipid material circulating was carried by the β-globulin fraction   Blix, Tiselius and Svensson in 1941 showed further that this and the σ-globulin fraction of the circulating proteins were especially rich in cholesterol and allied conjugate structures   Pathological states in which an increase of circulating sterols is known to occur might be expected to show an increase in these fractions, when the method used for the protein analysis is dependent, in part, on detection of refractive changes in the serum   Moreover, Gardner and Gainsborough (1930) have shown that increases in cholesterol may be expected in cases with obstruction to the outflow of bile

Patients, therefore, in whom there was clinical or laboratory evidence of gross obstruction have been excluded from this series   As a further precaution, where possible, direct analyses have been made of the total cholesterol content of sera actually analysed   All these analyses fell within normal limits   Out of the 18 analyses only two showed an increase in β-globulin beyond normal limits   This suggests that while β-globulin increases may occur they are not the rule in established uncomplicated cases

Longsworth, Shedlovsky and MacInnes (1939) have shown that in pyrexial patients an increase in the circulating σ-globulins may be observed   In the present series special care was taken to collect material from afebrile patients and, in only two analyses was the sum of the globulins above normal limits   Of the remaining 16, 12 fell below the mean of normal levels for the σ-globulins

In contradistinction there was an absolute and persistant increase in the circulating γ-globulin, persisting up to death   It has been asserted, frequently, that the increase in globulin is compensatory in nature   In the present series there is nothing to support this assertion   Thus γ-globulins of the order of 4 7 g per 100 ml have been observed within a few hours of death, as well as in patients relatively well compensated   Equally, levels of 1 3 and 1 6 g per 100 ml have been observed at the day of death and months before death

There is an accumulating body of evidence to indicate that γ-globulins in the healthy and the diseased are elaborated in the lymphoid and reticulo-endothelial tissues (Enders, J F , 1944 , Dougheity, T F , Chase, J H , and White, A , 1944 , and Martin, N H , 1947)   However, autopsies on patients dying from chronic liver failure, unlike those in acute infective hepatitis, do not
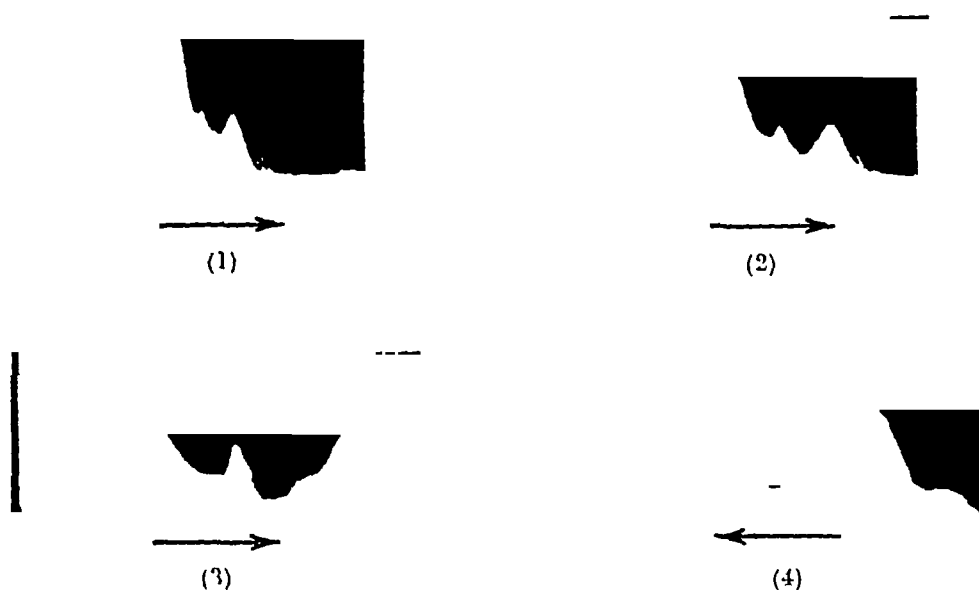

(1)

(2)

(3)

(4)

Fig   1 —Electrophoretic Analysis   Patient No 6   Arrows indicate the direction of migration
(1)   Descending Limb 1 hour
(2)   Descending Limb 2 hours
(3)   Descending Limb 3 hours
(4)   Ascending Limb 3 hours

show widespread hyperplasia of the lymphoid tissue nor of reticulo-endothelial system   Therefore the high levels of circulating γ-globulin observed in all these cases would seem to indicate a failure to dispose of, or modify, γ-globulin in the normal way   The fact that the liver is the primary site of failure in these cases suggests the possibility that it plays a part in the removal of γ-globulin in health This need not imply that it does more than alter the net charge by conjugation or other means so that it can no longer be identified as a γ-globulin   Nevertheless, such a modification could be of considerable biological significance

The peculiar behaviour of the γ-globulin in the analysis of Patient 6 deserves special note   Three hours from the start of the electrophoretic run, spontaneous precipitation was observed, localized in a well-defined band in the descending limb of the U-tube only (Fig 1)   All analyses were carried out at a protein concentration of 2 per cent , further concentrations of γ-globulin observed over

the series embrace that in which precipitation was observed   There was no sign of precipitation in the ascending limb   In the ascending limb of the U-tube the γ-globulin concentration is migrating in the presence of the other serum proteins, whereas in the descending limb the γ-globulin concentration is migrating in the presence of the buffer ions only   This would suggest that the associated circulating proteins have a stabilizing effect on globulins in solution

SUMMARY

(1) Serial electrophoretic analyses are presented from nine patients suffering from chronic progressive liver failure   In all cases there was a gross increase in the γ-globulin fraction and a marked decrease in the albumin fraction Spontaneous precipitation of γ-globulin was noted in one patient

(2) The β-globulin fraction in the majority of the analyses was normal or below normal limits

(3) The σ-globulin fraction in the majority of analyses was below the mean of normal levels

(4) Ancillary biochemical investigations carried out at the same time as the analyses are listed

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SURVEY OF PAPERS

ALBERT, RUBBO AND MARGARET I BURVILL have examined 80 heterocyclic bases, having flat rigid molecules, for antibacterial activity As with acridines, antibacterial activity is dependent on a high degree of ionization, and on the molecule having a minimal flat area Highly-ionized members of some series which have less than the minimum flat area acquire activity when a sufficiently large co planar area is built on to the molecule (p 159)

ASHESHOV, FRIEDA STRELITZ AND ELIZABETH A HALL have found two antibiotic substances, produced by an *Aspergillus* sp , which are active against certain bacteriophages They describe experiments which indicate that the antiphage substances interfere with the multiplication or formation of the phage corpuscles inside bacteria (p 175)

FISHER, continuing his studies on the inhibition of pertussis haemagglutinin by the acetone soluble lipoids of erythrocytes, finds that the inhibitory power is due to a sterol, probably cholesterol In saline suspensions the sterol fraction must be accompanied by the saponifiable fraction of the lipoids, in order to exhibit its inhibitory power to the full In model experiments the erythrocyte sterols may be replaced by cholesterol, and the saponifiable lipoids by lecithin or oleic acid (p 185)

WAINWRIGHT AND POLLOCK find that the nitratase preformed in bacteria that have been adapted to growth in the presence of nitrate remains unmodified during growth in a nitrate free medium so that the enzyme activity per bacterial cell gradually falls to its normal pre adaptive level (p 190)

GRUBB AND MORGAN find that there is a close association between the " Lewis " blood group character Le(a +) and inability to secrete A-, B and H substances Le^a- and Le^b-substances are present in secretions of persons of the appropriate genotype and show the same general physical and chemical characters of the A-, B and H-substances (p 198)

GILLIVER, HOLMES AND ABRAHAM describe an antibiotic substance, alvein, which is a strongly basic polypeptide produced by a strain of *B alci*, and is highly active against Gram-positive bacteria and less active against Gram-negative bacteria (p 209)

GILLIVER has tested 165 strains of aerobic spore bearing bacilli for their ability to secrete antibacterial substances into lemco agar and into potato dextrose broth All the strains of *B subtilis*, *B pumilus* and *B licheniformis* are active, and only one third of the other strains are active (p 214)

LUNDBACK has found that the complement-fixation test on sera of 60 mumps patients gave results in agreement with the clinical findings, whereas the haemagglutination inhibition test failed to reflect the actual antibody content of the sera (p 221)

MARTIN presents serial electrophoretic analyses of the blood serum from 9 patients suffering from chronic progressive liver failure In all cases there was a gross increase in the γ-globulin fraction and a decrease in the albumin fraction The β globulin fraction was normal or below normal, and in most cases the globulin fraction was below the mean of normal levels (p 231)

STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID IN-HIBITING INFLUENZA VIRUS HAEMAGGLUTINATION PRE-CIPITATION-DISSOLUTION REACTION IN MIXTURES OF ACTIVE VIRUS AND INHIBITOR

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IN previous papers (Svedmyr, 1947a, 1947b, 1948a, 1948b, Gard, von Magnus and Svedmyr, 1947) the haemagglutination inhibitor was described which is present in normal allantoic fluid obtained from chick embryos   The observations indicated that the inhibitive capacity of normal allantoic fluid on influenza virus haemagglutination is connected with particles having a sedimentation constant of about 200 S, and that the inhibitor acts by combining with the virus particles   In high concentrations the reaction manifested itself as a macroscopic flocculation

Active virus, even in small quantities, was shown to have the capacity, when incubated at 35° C, with normal allantoic fluid, to destroy gradually the inhibitory activity   After sufficient destruction, or modification, of the inhibitor particles, haemagglutinins appeared in the previously non-haemagglutinating mixture

By suitable treatment it was possible to destroy successively the capacity of the virus particle (a) to infect, (b) to modify the inhibitor, and (c) to combine with the inhibitor and to haemagglutinate

Certain of these previously reported results have been confirmed by the work of Hardy and Horsfall (1948)

The purpose of this investigation was to study the virus-inhibitor precipitation in some detail

MATERIALS AND METHODS

Virus Preparations

The PR8 strain of influenza virus was used throughout these experiments   The virus was propagated in the allantoic cavity of 11-day-old chick embryos   Two types of virus preparations were employed   the classical " standard virus "

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(SV) with a sedimentation constant of about 700S, and the "modified virus" (MV) obtained by three allantoic passages of undiluted allantoic fluid, its main component having a sedimentation constant around 500 S (von Magnus, 1947*a*, 1947*b*, Gard and von Magnus, 1947, Gard, von Magnus and Svedmyr, 1947, Svedmyr, 1948*b*)

Purification

Virus concentrates were prepared by differential centrifugation in two cycles of allantoic fluid clarified by suction-filtration through tightly-packed cotton-wool   The first cycle included sedimentation and washing in a refrigerated Sharples centrifuge operating at 48,000–50,000 r p m with a rate of flow of about 1 4 litres per hour, followed by clarification in an angle centrifuge at 5000 r p m for 5 minutes   The second cycle consisted of sedimentation in an air-driven centrifuge at 27,000 r p m for 20 minutes, and a further low speed centrifugation at 5000 r p m for 5 minutes   The final virus concentrate was made up with phosphate buffer to 1/1000 of the starting volume of allantoic fluid

In the MV preparation used this general procedure was slightly modified The clarification of the Sharples concentrate was performed by centrifugation in an " Ecco," bucket-type centrifuge at 4500 r p m for 10 minutes, the second high speed centrifugation was run for 90 minutes instead of 20, and the final clarification was performed by spinning up-and-down to 10,000 in the air-driven centrifuge   The concentration by volume was about 900 times that of the starting material   This preparation was kindly supplied by Dr P von Magnus

The nitrogen contents of the preparations used in the present experiments were as follows   SV1—4 3 mg per ml , SV2—4 0 mg per ml , MV—1 48 mg per ml   The biological properties of SV1 and MV are listed in Tables I and II

Table I shows that SV1 contained 50 per cent or more of the activity of the starting material, as measured both by infectivity, haemagglutination titre and inhibitor-destroying capacity   The term " active virus " means virus that has retained its capacity to destroy the inhibitor, the infectivity being irrelevant in this connection

Inhibitor Preparations

Two preparations of inhibitor were used in these experiments   The starting material was one batch of normal allantoic fluid from embryos 13 days old and one pool from embryos 14 days old   The purification methods were essentially the same for the two batches   After clarification the fluid was diluted to about double the volume with buffered saline in order to minimize precipitation of uric acid during the next step   The fluid was then concentrated to about 1/25 of the original volume by means of ultrafiltration   The concentrate was subjected to two cycles of differential centrifugation   First, 27,000 r p m for 90 minutes, followed by clarification in an angle centrifuge at 5000 r p m for 10 minutes for Batch 1 and spinning up-and-down to 10,000 r p m in the air-driven centrifuge for Batch 2 , then a further 60 minutes at 27,000 r p m , and a final low speed centrifugation at 5000 r p m for 15 minutes   The final concentrate was made up with phosphate buffer to 1/1000 of the original volume   Both preparations contained 0 51 mg nitrogen per ml   Titres of the inhibitory activity of the different fractions are listed in Table III

TABLE I.—*Properties of the Various Fractions from the Differential Centrifugation of SV Preparation 1*

Virus material	Allantoic fluid				Supernatant from Sharples centrifugation		Sharples sediment		Supernatant 5000 I	Control without virus
50 per cent chick embryo infectivity endpoint (3 eggs per group)	7·8				2·93		5·36		5·44	0·98
Haemagglutination titre	4·00									
Residual inhibitory activity of normal allantoic fluid incubated with the virus* — Concentration of virus	1/100	1/300	1/900	1/2700	10^{-1}	10^{-2}	10^{-2}	10^{-1}	10^{-2}	
Hours of incubation 0	0·83	1·06	0·98	1·13	0·91	0·98	0·45	1·06	0·38	
Hours of incubation 3	0·45	0·91	0·98	1·13	0·53	0·98	+	0·91	+	
Hours of incubation 20	+	0·38	0·68	0·98	+	0·75	+	0·30	+	

+ = Positive haemagglutination of the tested fluid

* Normal fluid was incubated at 35° C with different amounts of the virus. Samples were removed after various periods of time and tested for residual inhibitory activity after heating in a 65° C water bath for 15 minutes.

TABLE II.—*Properties of the MIV Preparation*

Virus material	"3 passage" allantoic fluid	Sediment 5000 I	Supernatant 27,000	Sediment 27,000	Supernatant 10,000 = final concentrate			Supernatant 5000 II = conc. 11·5		Sediment 5000 II	Control SV Preparation 1			Control without virus
50 per cent chick embryo infectivity endpoint (3 eggs per group)	4·9	4·51	2·78	0·71	6·9			0·78		4·99				0·98
Haemagglutination titre	3·33				5·97									
Residual inhibitory activity of normal allantoic fluid incubated with the virus* — Concentration of virus		10^{-2}			1/10,000	1/40,000	1/160,000	10^{-3}	10^{-4}		1/50,000	1/200,000	1/800,000	
Hours of incubation 0		0·08			0·83	0·98	0·98	0·98	1·06		0·83	1·06	1·06	
Hours of incubation 3		+			0·38	0·38	0·68	0·83	1·13		−0·07	0·83	1·06	
Hours of incubation 20		+			0·08	0·23	0·38	+	0·98		+	−0·07	0·38	

+ = Positive haemagglutination of the tested fluid

* See note, Table I.

TABLE III —*Titie Values of the Inhibitory Activity of the Various Fractions from the Purification of Inhibitor*

Inhibitor fraction	Inhibitory activity *	
	Preparation 1	Preparation 2
Original normal allantoic fluid	1 43	1 13
Same diluted by phosphate buffer	1 13	1 13
Ultrafiltrate	—0 07	—0 07
Ultraconcentrate .	0 83	
Supernatant 27,000 I	0 23	0 38
Sediment 27,000 I	0 53	
Supernatant 5000 I	0 53	
Sediment 5000 I or 10,000 respectively	—0 07	0 23
Supernatant 27,000 II	—0 07	0 08
Sediment 27,000 II	0 75	0 53
Supernatant 5000 II	0 83	0 83
Sediment 5000 II	0	0 08

* The inhibitory activity was determined in the usual way after dilution of the test material corresponding to the volume of the starting material

Most of the preparations and reaction mixtures from the present experiments were analysed in the ultracentrifuge   The results will be reported in detail elsewhere   It should only be mentioned that the SV and MV appeared pure in the centrifuge   The inhibitor preparations in addition to the "200 S" component contained a homogeneous slow-sedimenting component ("30 S")

Preservatives

The use of merthiolate, employed in earlier experiments (Svedmyr, 1948a, 1948b) as preservative, has been found to cause a certain loss of virus activity during purification   Instead, 100 units of crystalline penicillin and 100 γ of streptomycin per ml   was added to all concentrates

Buffer solutions

0 1 M phosphate buffer with a pH of 7 0 was used   Inhibitor Concentrate 2, however, was taken up in a mixture of 80 per cent saline and 20 per cent of this buffer

Saline

0 9 per cent NaCl in distilled water was used

Titration Methods

Haemagglutination tests

The methods were described in detail and their accuracy evaluated in a previous paper (Svedmyr, 1948a)   All titre values are recorded as the logarithm of the final virus dilution

Inhibitory activity —Red cells from a minority of fowls gave lower titres when used in the titrations for inhibitory activity, which agrees with the observations

of Anderson (1948) In these experiments only cells giving high inhibitor titres were used

The inhibitory activity of a test material was usually determined as previously reported (Svedmyr, 1948a), i e serial dilutions of an active standard virus allantoic fluid were made up with the inhibitor material as diluent, and the difference between the log titre value obtained and the control titre of the virus when diluted in saline was recorded

In some experiments, however, where very many titrations had to be performed on the same day, a somewhat modified technique was employed To 0 25 ml amounts of the inhibitor material 0 25 ml of each virus dilution was added and, finally, 0 5 ml of red cell suspension Stock dilutions of virus could thus be prepared at one time for a series of titrations When corrected for different virus and inhibitor amounts no significant difference in titres was observed with the two methods, i e within the range of activity employed in the present experiments

Inhibitor-destroying capacity —The inhibitor-destroying capacity of a virus suspension was tested as reported previously (Svedmyr, 1948b) , normal allantoic fluid was incubated at 35° C with small amounts of the virus Samples were removed after different periods of time and tested for residual inhibitory activity after being heated in the water bath at 65° C for 15 minutes in order to interrupt the process

Chick embryo titrations

Chick embryo titrations were performed as previously described (Svedmyr, 1948b)

EXPERIMENTAL

It was previously reported (Svedmyr, 1947b, 1948b) that the precipitate appearing in a mixture of purified concentrates of influenza virus and inhibitor seemed to be rather stable even after incubation at 35° C for one hour, and it was assumed that the virus suspension had lost most of its inhibitor-destroying capacity, possibly since it had been stored for a long time with 1/10,000 merthiolate as preservative As a matter of fact, quite different results were obtained with the active virus preparations described here

In the present experiments the volume of the reaction mixtures varied from 2 5 ml in tests intended for analysis in the ultracentrifuge and for haemagglutination to 0 05 ml , which was found sufficient for observation of the flocculation with the aid of a magnifying-glass When mixtures had to be prepared at low temperatures reagents as well as glassware were pre-chilled

Precipitation phase

Under the conditions mentioned the SV preparations formed heavy precipitates when mixed with inhibitor in the proportions 1+4, leaving an almost clear supernatant after storage in the cold The MV preparation, being less concentrated, gave a rather slight precipitate when mixed in the same proportions, presumably with free inhibitor in the supernatant When mixed with inhibitor in equal amounts, however, the MV concentrate also gave a heavy flocculation and a comparatively clear supernatant

Dissolution phase

When the tubes containing these heavy precipitates were placed in an incubator at 35° C or only warmed in the hand, the suspension cleared up almost completely in about 2 minutes, and after 5–10 minutes no floccules could be seen with a magnifying-glass   In the first 5 minutes in the incubator the temperature in the tubes may have reached 30° C

The behaviour of mixtures with SV concentrates was studied in some detail

Repeated cycles of combination-elution with the same virus amount

It is well known (Hirst, 1942) that influenza virus can be adsorbed to and eluted from red cells several times without apparent change of its properties The experiment below indicates that the interaction between virus and inhibitor of crude allantoic fluids (inhibition→inhibitor destruction) may also be repeated several times by successive addition of fresh normal allantoic fluid, the haemagglutinins being released almost quantitatively each time

A twofold serial dilution in saline was made of a virus-infected allantoic fluid Portions of the dilutions were further treated in two ways

(1) Each virus dilution was mixed with an equal amount of normal allantoic fluid, incubated at room temperature for 30 minutes, then chilled in ice-water, after which the haemagglutination titre of each mixture was determined (Curve A in Fig 1)

(2) Control, treated in the same way, but with saline substituted for normal allantoic fluid (Curve B in Fig 1)

Obviously a good deal of the haemagglutination of the mixtures with the lowest concentrations of virus was still inhibited after 30 minutes of incubation at room temperature

The experiment was repeated in the same way, but with 20 hours of incubation at 35° C before titration (Fig 2)   After this incubation there existed no signs of inhibition even with the lowest virus concentrations

New amounts of normal allantoic fluid and saline, respectively, were added to each dilution of the last-mentioned experiment, and the haemagglutination titres were determined after a further incubation at 35° C for 20 hours (Fig 3)

There was now a significant drop in the titres of the lowest virus concentrations   However, the control showed the same decrease of titre, suggesting that the smaller maximal " elution " of haemagglutinins in mixtures with low virus concentrations might be only an artefact produced by a more rapid inactivation of haemagglutinins when in these concentrations

It may be pointed out that Hardy and Horsfall (1948) in similar experiments apparently did not include controls, and they do not mention the possibility of an inactivation of haemagglutinins as a cause of the drop in titre of the low virus concentrations   Moreover they seem to underestimate the necessity of increasing the incubation time to obtain maximal release of haemagglutinins in mixtures with low concentrations of virus   The validity of their conclusions regarding the existence of bound virus " in virus-infected allantoic fluid is, therefore, still uncertain

That the precipitation-dissolution phenomenon can also be repeated with a single virus aliquot by successive additions of fresh inhibitor is shown by the following experiment

Active SV Concentrate 2 (0 03 ml) was mixed with the same volume of inhibitor Preparation 1 in a small test-tube   The reagents were kept in ice-water A precipitate immediately appeared with large floccules adhering to the walls After being kept in ice-water for 2½ hours the tube was incubated at 35° C for 15 minutes   The precipitate was dissolved in about 2 minutes   The tube was
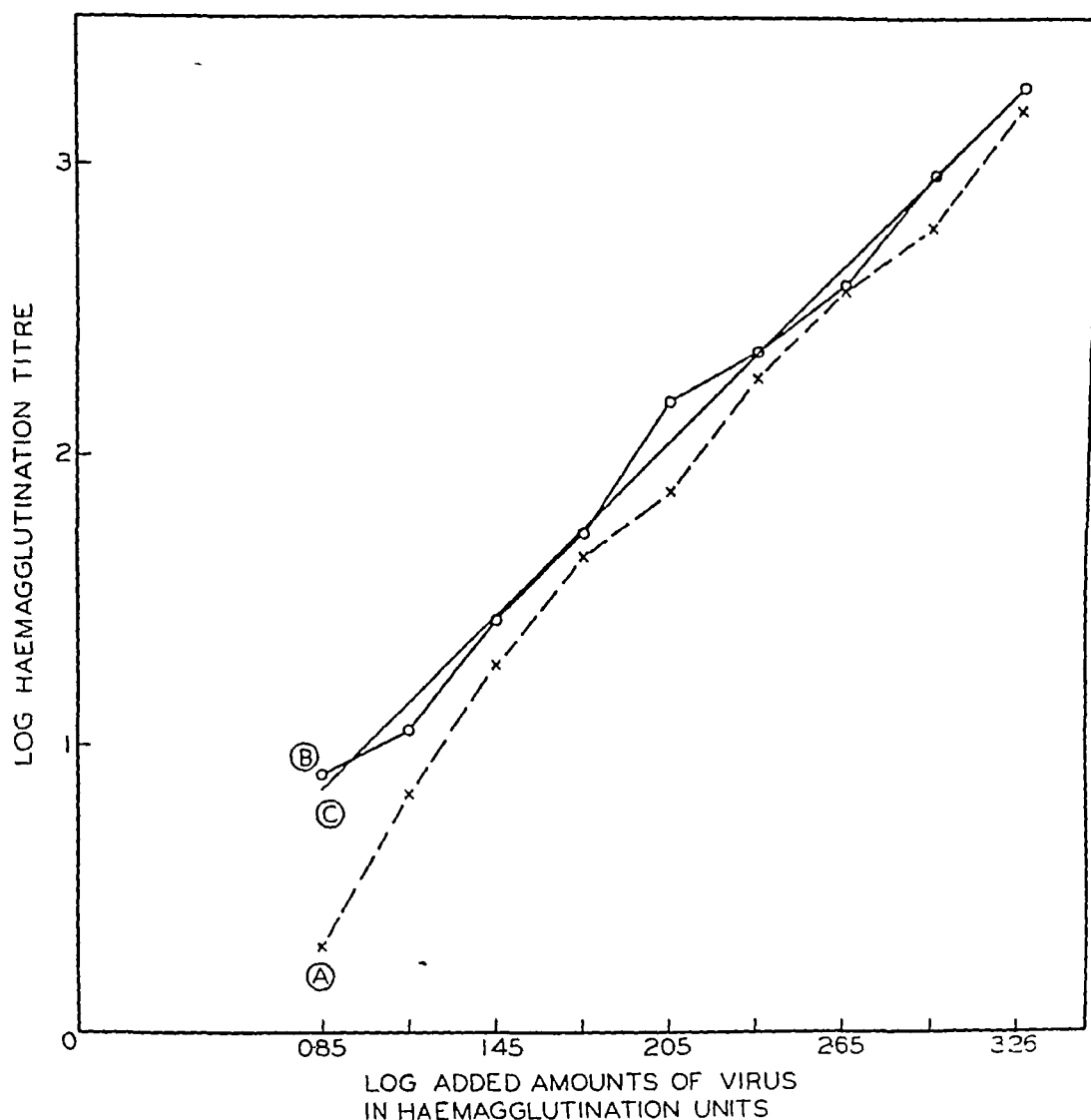

Fig 1 —Free haemagglutinins in virus—normal allantoic fluid mixtures after incubation for 30 minutes at room temperature   (A) x – – x, Virus diluted in saline + normal allantoic fluid   (B) o——o, Virus diluted in saline + saline , (c) ——, Haemagglutination titres calculated from control containing highest virus concentration

again chilled in the ice-water for 10 minutes, the suspension remaining quite clear   After this the whole precipitation-dissolution cycle was repeated three times by adding further aliquots of inhibitor under similar conditions   The reaction ran the same course each time, although the precipitate was a little less flocculent in the last cycles, perhaps depending upon the higher dilution

Influence of temperature on the dissolution time

As mentioned above, virus-inhibitor precipitates, formed in the cold, were completely dissolved in a few minutes when brought into a 35° C incubator   If, on the other hand, the reagents and the mixture were kept at room temperature during the whole operation, only a turbidity appeared immediately and cleared
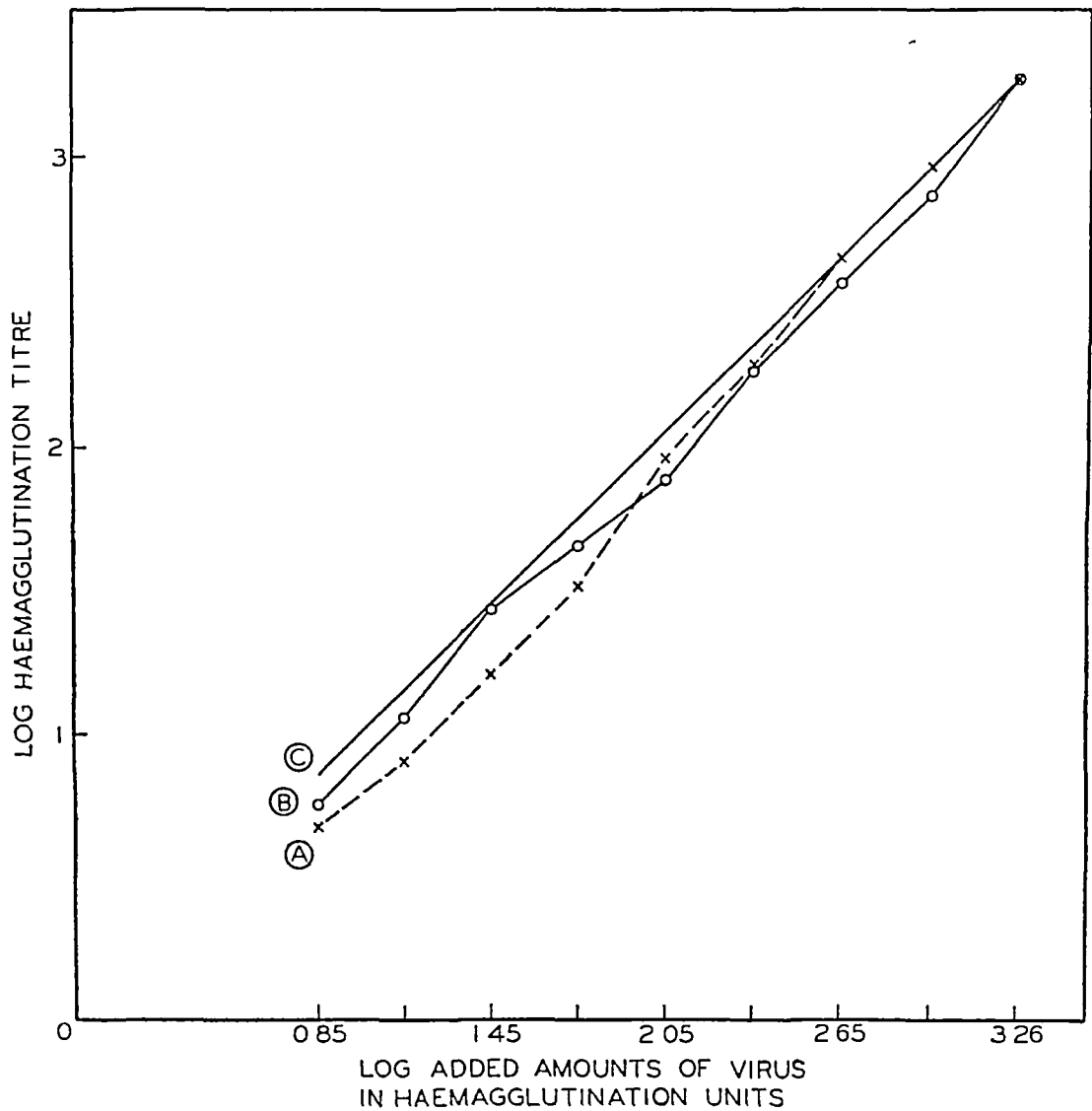

Fig 2 —As in Fig 1, but incubation for 20 hours at 35° C

up in a few moments, showing that the dissolution reaction, at these high concentrations, proceeds very rapidly   At 0 to 1° C, however, even 2½ hours' incubation brought about no visible reduction in the precipitate

A reaction rate intended to make observation more convenient was established in an experiment performed in a water bath kept at 10 5–11 0° C   Active SV Concentrate 1 (0 15 ml) was mixed with inhibitor (0 6 ml Preparation 1) at a temperature of 5° C   A heavy precipitate appeared immediately   The tube

was then kept in the water bath during the whole experiment   It was found that the precipitate was not altered in the first 15 minutes , at 45 minutes a slight reduction was apparent, and after 2 hours and 20 minutes only very slight residues of the floccules could be seen with the magnifying-glass, being completely dissolved a few minutes later (Table IV)
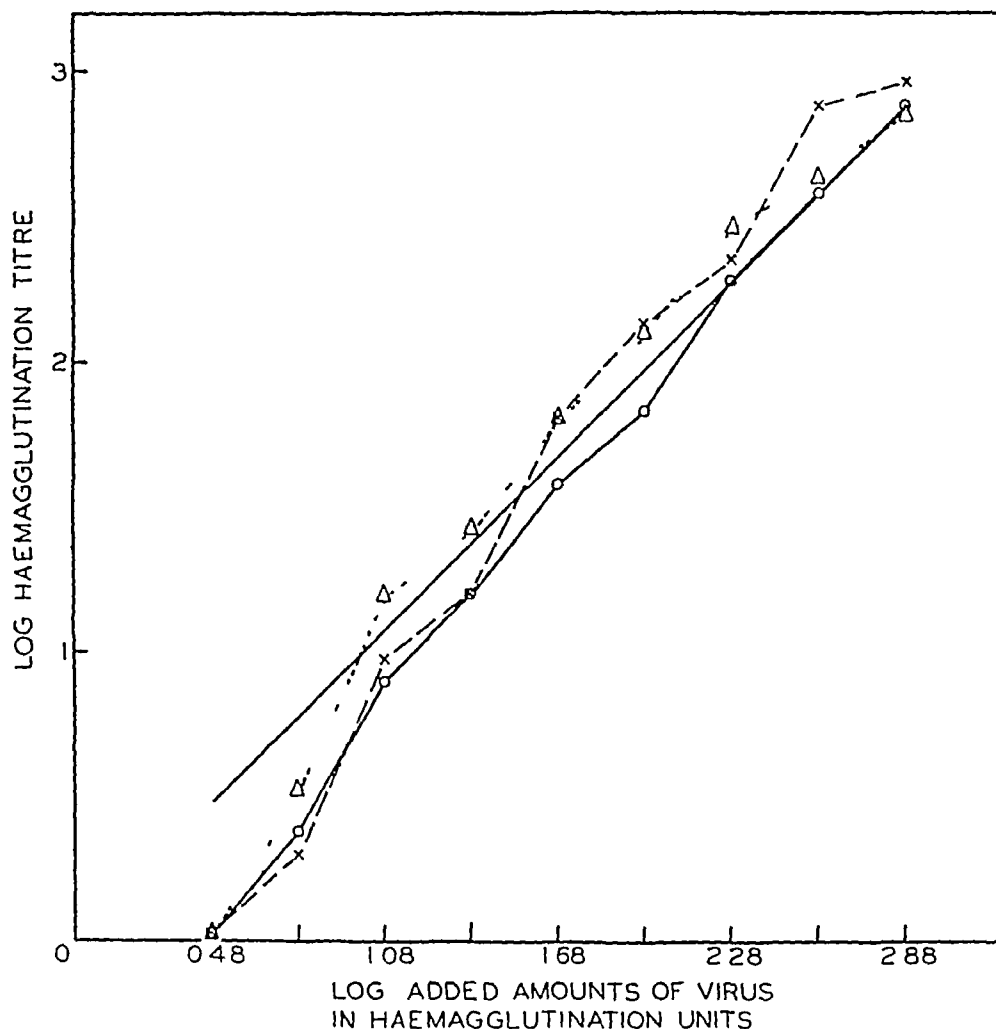

FIG 3 —Free haemagglutinins in virus—normal allantoic fluid mixtures after incubation for 20 hours at 35° C with repeat after addition of further allantoic fluid △ △, Virus diluted in saline + normal allantoic fluid, incubation for 20 hours at 35° C , repeat after further addition of normal allantoic fluid   x - - x, Virus diluted in saline + saline, incubation for 20 hours at 35° C , repeat after addition of normal allantoic fluid   o——o, Virus diluted in saline + saline, incubation for 20 hours at 35° C , repeat after further addition of saline ——, Haemagglutination titres calculated from control containing highest virus concentrations

Correlation between the precipitation-dissolution phenomenon and the release of haemagglutinins

Some of the precipitation-dissolution experiments described above were also analysed by haemagglutination titrations   0 1 ml samples were removed after different periods of time, immediately heated in a water bath at 65° C for 15

TABLE IV —*Haemagglutination Titres and the Disappearance of Precipitates on Incubation of Mixtures of 4/5 of the Inhibitor Preparation 1 and 1/5 of the SV Preparation 1*

Incubation temperature		Duration of incubation	Haemagglutination titre of the mixture *	Approximate gradation of the precipitate as seen through a magnifying-glass
35° C	{	0	4 16	+++†
		3 hrs	5 26	—
	{	48 hrs	5 56	—
10 5°–11 0° C	{	0	4 16	+++
		5 mm	4 39	+++
	{	15 ,,	4 54	+++
	{	45 ,,	4 69	++
		2 hrs 20 mm	4 99	(+)
	{	4 hrs	5 ˙14	—

* Samples were removed after the indicated periods of time, inactivated in the 65° C water bath for 15 minutes to stop the process and then kept at 4° C until titrated

† When mixed at + 1° C a heavy precipitate was formed which at 35° C disappeared in about 3 minutes

Control , The virus mixed with phosphate buffer instead of inhibitor and then likewise heated at 65°C for 15 minutes   Titre 5 71

minutes in order to interrupt the process, and then stored at 4° C until titrated A virus control with phosphate buffer substituted for inhibitor and treated in the same way was included   Two such experiments with the SV Preparation 1 are listed in Table IV

It is obvious that the macroscopically visible precipitate had disappeared before the haemagglutination reaction had reached its maximum   The titre, even after 48 hours of incubation, was less than the control, but the difference is not significant   The experiment has been repeated with a similar result   It is, however, impossible to judge from the available data whether the release of virus haemagglutinins from the complex is complete

The elution of haemagglutinins from a mixture of MV and inhibitor was tested with the same methods   It appeared, however, that these haemagglutinins were almost quantitatively destroyed by the heat treatment that was used to interrupt the elution process, even in the control without inhibitor   Whether this depends on a constant difference in heat resistance between standard virus and modified virus remains to be investigated   It may be mentioned that some older SV concentrates also did not resist the same heat treatment

However, if titrated without previous heat inactivation the MV-inhibitor mixture reached a titre of 5 31 after incubation at 35° C for 48 hours, which should be compared with the virus control titre of 5 23, i e no significant difference

SUMMARY

The interaction between the PR 8 strain of influenza virus and the haemagglutination inhibitor of normal allantoic fluid, both purified and concentrated by two cycles of differential centrifugation, has been studied

Active virus, which, in this connection, means virus that has retained its capacity to render the inhibitor particles non-inhibitive, immediately gives a visible precipitate when mixed with the inhibitor under suitable conditions The precipitate is then gradually dissolved, whereby free virus haemagglutinins reappear The precipitation-dissolution phenomenon can be repeated several times with a single aliquot of virus by successive additions of fresh inhibitor

The disappearance of the precipitate at these high concentrations proceeds at a fairly rapid rate—at about 30° C in a few minutes, at 11° C in about 1 to 2 hours

From the available results it is not possible to judge whether virus is completely eluted from the virus-inhibitor complex

Both the classical " standard virus " with a sedimentation constant of about 700 S and the " modified virus " with a sedimentation constant at around 500 S give this precipitation-dissolution phenomenon

As a whole the precipitation-dissolution reaction reflects the course of the modifying effect of normal allantoic fluid on influenza virus haemagglutination

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION PRECIPITATION REACTION IN MIXTURES OF INACTIVE VIRUS AND INHIBITOR

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THE preceding paper (Svedmyr, 1949) described the formation and dissolution of precipitates in mixtures of purified concentrates of active influenza virus (PR 8) and the inhibitor present in normal allantoic fluid from chick embryos

In the present paper it will be shown that precipitates obtained with properly pre-heated virus do not dissolve spontaneously   Data regarding the composition of the stable precipitate will be given   Furthermore, the influence of virus-antibodies on the precipitation reaction is described

MATERIALS AND METHODS

Heat-inactivated virus preparations

The virus was inactivated as follows   Flasks containing 100 ml of the preparations were immersed in a 65° C water bath for 15 minutes   The temperature of the material exceeded 60° C for about 10 minutes   The first preparation (heated " standard virus " Preparation 1 = HSV 1) was made from part of the final active preparation SV 1 described in the preceding paper (Svedmyr, 1949) This was diluted 1/50 before heating and afterwards reconcentrated about 50 times by centrifugation in the air-driven centrifuge at 27,000 r p m for 20 minutes On another occasion (Preparation HSV 2) a fraction of a clarified Sharples concentrate (SV 2) was inactivated and then further purified as previously described for the active virus

The nitrogen content of the inactive virus concentrates was 3 3 (HSV 1) and 1 8 (HSV 2) mg per ml , the haemagglutination titres 6 48 and 6 71 respectively

Inhibitor preparations

In addition to the preparations described in the preceding paper (inhibitor Preparations 1 and 2 Svedmyr, 1949) a third concentrate was employed   The starting material was normal allantoic fluid from 14-day-old chick embryos and the purification methods were essentially the same as for Preparation 1   The final concentrate contained 0 75 mg of nitrogen per ml , and showed an inhibitory activity of 0 83 when titrated in a dilution of 1/1000, as compared with 1 58 for the starting material

Anti-PR 8 serum

Serum from a ferret convalescent after infection with PR 8 influenza virus was used   Before use the serum was heated to 56° C for 30 minutes   The potency of the serum was checked by titration for complement-fixing capacity

Capillary centrifuge tubes

For quantitative estimation of the precipitates the reaction mixtures were centrifuged in special tubes, where the sediment was collected in gauged glass capillaries   The volume of the precipitate was estimated from the height of the closely packed sediment.   Two sets of tubes were used, one type holding about 0 18 ml , the other, used for diluted preparations, holding 5 ml   The capillary of the second type was graded, that of the former was ungraded, the height of the sediment in that case being determined with calipers   With wooden adapters the tubes could be placed in the buckets of an International Size 3 centrifuge

The composition of buffer solutions and saline as well as a description of the titration methods is given in the preceding paper (Svedmyr, 1949)

EXPERIMENTAL

Precipitation with heat-inactivated virus

Heating to 56° C for 30 minutes is known to inactivate the " enzymatic " effect of influenza viruses on the red cell receptors (Briody, 1948 , Hirst, 1948*b*), as well as on the inhibitors of normal serum (Hirst, 1948*b* , Burnet, 1948) and normal allantoic fluid (Svedmyr, 1948)   It was, therefore, to be expected that virus-inhibitor precipitates obtained with heated virus should not show the phenomenon of spontaneous dissolution, provided the virus " enzyme " is responsible for this reaction

The validity of this assumption was tested in the following experiment

Heat-inactivated virus (HSV 1) was mixed with inhibitor (Preparation 1) in the proportions 1 to 5 at room temperature   A flocculent precipitate appeared, remaining unchanged during a further incubation at 35° C for 48 hours   Titrations for haemagglutinins showed no difference between samples taken immediately after mixing, after 3 and after 48 hours at 35° C   The titres were 1 68 as compared with the virus control 5 71, indicating that no virus was eluted during incubation

Quantitative data on the virus-inhibitor precipitation

The formation of a stable precipitate in mixtures of HSV and inhibitor made it possible to collect quantitative data regarding the precipitation reaction   An attempt was made to study to what extent the amount of precipitate and the activity of the supernatant were dependent upon the concentrations of the reactants

In the special centrifuge tubes mixtures were prepared of constant amounts of one preparation with aliquots of serial dilutions of the other, measured by means of capillary pipettes or a microburette   A control containing only virus and phosphate buffer was included in each series   The precipitates were observed under a magnifying-glass and their appearance recorded, after which the tubes were left at room temperature for about 4 hours , or, in the first experiment, for

30 minutes and then at 4° overnight The tubes were then spun in a bucket type centrifuge The sediments were measured and the supernatants removed for haemagglutination tests

It appeared that in high concentrations the sedimentation of the precipitate was delayed in tubes with excess of one of the components, as could be expected from the high viscosity of the preparations the greater the excess, the slower the sedimentation Therefore, after removal of the supernatants the sediments were further centrifuged until the volumes appeared constant In the last experiment the sedimentation disturbances caused by viscosity variations were minimized by diluting the mixture 1/100 in phosphate buffer before centrifugation

The results of two typical experiments are indicated in Tables I and II and Fig 1 and 2

TABLE I —*Variation in the Virus-inhibitor Precipitate with Varying Concentrations of Inhibitor   Constant Quantity of Virus   HSV 2–0 02 mg Nitrogen per Tube*

Added amount of inhitor Preparation 2 in mg nitrogen	Approximate gradation of the immediate flocculation	Height in mm of the sediment after centrifugation for 3½ hr at 1700 r p m and 1 hr at 2400 r p m	Haemagglutination titre of the supernatant after 1½ hr at 1700 r p m
0 041	+++	6 0 still not homogeneous	<2 09
0 041/2	+++	4 4 still not homogeneous	<1 89
0 041/4	+++	3 7	<1 89
0 041/8	+++	2 9	<1 89
0 041/16	+++	2 2	1 89
0 041/32	+++	1 5	2 64
0 041/64	+++	1 4	3 69
0 041/128	++	1 1	4 14
0 041/256	(+)	0 9	4 52
0 041/512	—	0 9	4 67
0	—		4 82

TABLE II —*Variation in the Virus-inhibitor Precipitate with Varying Concentrations of Virus   Constant Quantity of Inhibitor Preparation 3–0 0038 mg Nitrogen per Tube, except the Control   Before Centrifugation the Mixture was Diluted 1/100 in Phosphate Buffer*

Added amount of HSV 2 in mg nitrogen	Approximate gradation of the immediate flocculation	Volume of the sediment in mm^3 after 4½ hr at 2800 r p m	Hemagglutination titre of the supernatant after 30 min at 2800 r p m
0 17	+++	6 0	3 16
0 17/2	+++	2 9	2 56
0 17/4	+++	1 3	1 28
0 17/8	++	0 6	0 30
0 17/16	+	ca 0 3	<0 30
0 17/32	—	ca 0 1	<0 30
0 17, no inhibitor	—	2 0	3 91

An experiment in which decreasing amounts of inhibitor were added to constant amounts of virus (Table I, Fig 1) showed that haemagglutinins appeared in the supernatant when the original concentration of inhibitor was below a certain level   A closer analysis reveals that the amount of virus fixed in the
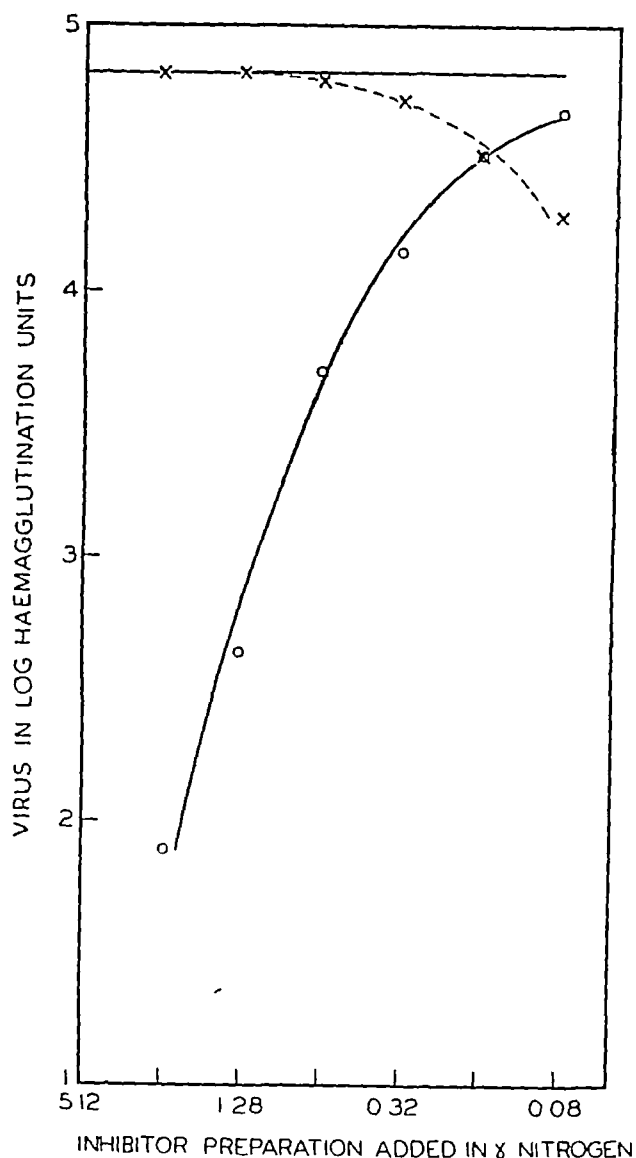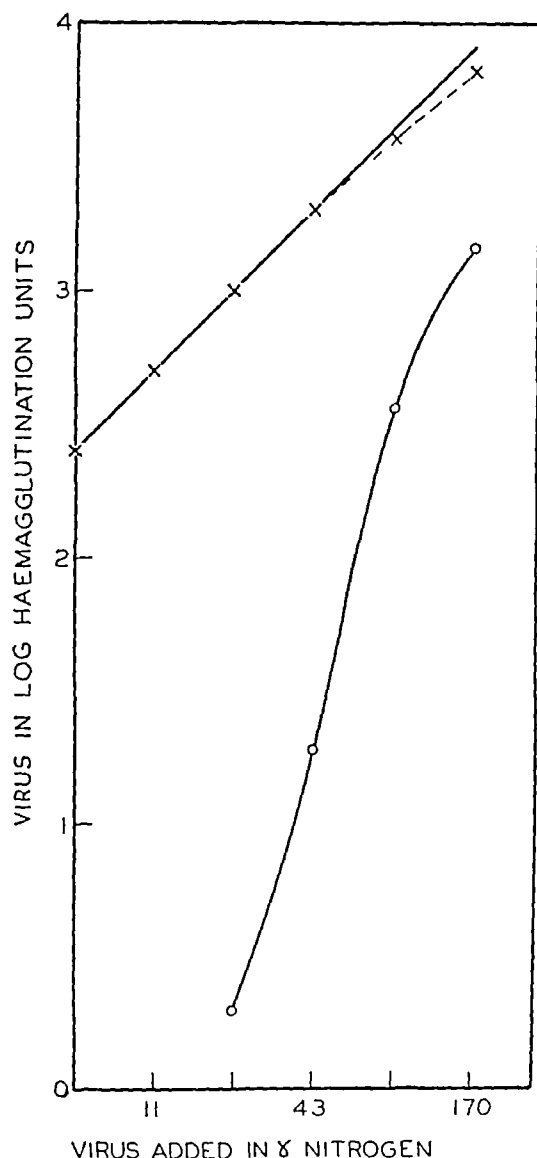

INHIBITOR PREPARATION ADDED IN ɣ NITROGEN

Fig 1

VIRUS ADDED IN ɣ NITROGEN

Fig 2

Fig 1 —Variations of free and bound virus in mixtures with varying concentrations of inhibitor
o———o = Free virus , x – – – x = bound virus , ——— = added virus

Fig 2 —Variations of free and bound virus in mixtures with varying concentrations of virus
o———o = Free virus , x – – – x = bound virus , ——— = added virus

precipitate is not proportionate to the quantity of inhibitor   Under the given conditions proportionately more virus is bound the smaller the amount of inhibitor used   The results of haemagglutinin titration and measurement of precipitate volume coincide largely in this respect   However, by direct observation of the

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newly formed precipitate it was impossible to detect significant differences in the appearance of the precipitate, except in tubes with very small amounts of inhibitor Direct observation, therefore, cannot be relied upon for quantitative estimations

The results recorded in Table II and Fig 2 are in keeping with those of the previous experiment   The more virus is added to a certain amount of inhibitor the larger is the quantity incorporated in the precipitate

The figures in Table I seem to indicate that the reverse may also be true   If so, the inhibitor, when added in excess, seems to be less readily incorporated Further experiments, including titrations for inhibitory activity, are necessary before this question can be answered

Inhibition of precipitation reaction by specific PR 8 antiserum

The effect of anti-serum against PR 8 virus on the virus-inhibitor precipitation was tested in the following way

PR 8 ferret serum (0 20 ml) was treated with 0 05 ml of $M/10$ $NaIO_4$ (final dilution $M/50$) at room temperature for 2 5 hours in order to eliminate the effect of the normal serum inhibitor (Hirst, 1948a)   The excess $NaIO_4$ was then destroyed by adding 0 05 ml of 10 per cent glucose

Three tests were performed

(1) 0 01 ml of heat-inactivated standard virus was mixed with 0 09 ml of the periodate-treated serum and kept at room temperature for 30 minutes   The mixture was clear   After that 0 04 ml of inhibitor was added   No precipitate appeared

(2) Inhibitor and virus were mixed first, the typical flocculent precipitate appearing   After 30 minutes at room temperature the serum was added   The precipitate remained unchanged

(3) Virus was diluted with phosphate buffer instead of serum, inhibitor added last   A typical precipitate appeared

The three tubes were then incubated at 35° C   After one day a very slight deposit appeared in the first tube, while in Tubes 2 and 3 the precipitates had settled into heavy sediments, the supernatants remaining rather clear   The appearance was quite the same throughout 4 days of incubation

Obviously the immune serum could prevent the virus-inhibitor precipitation even if it were added in a concentration forming only insignificant amounts of immuno-precipitate when mixed with the virus   However, the same amount of virus-antibody was not able to displace the inhibitor in the combination with virus, at any rate not enough to dissolve the precipitate

DISCUSSION

The results reported above clearly indicate that the composition of the virus-inhibitor precipitate varies with the original concentrations of the components On the basis of available data a calculation of the composition of the precipitate was attempted

The ratio of inhibitor to virus particles may be computed as follows

$$\frac{\text{Number of inhibitor particles}}{\text{Number of virus particles}} = \frac{N_i}{N_v} \cdot \frac{h}{} \cdot \frac{s_v^3}{s_i^3} \cdot \frac{(1 - F_i)}{(1 - F_r)},$$

where

N_i = amount of inhibitor preparation nitrogen added,

N_v = amount of virus nitrogen added,

k = correction factor for impurities (" 30 S ") present in the inhibitor preparation. It was calculated from ultracentrifugation data

s_i = the sedimentation constant of the inhibitor particles (= 200 S),

s_v = the sedimentation constant of the standard virus particles (=700 S),

F_i = fraction of total inhibitor remaining uncombined,

F_v = fraction of total virus remaining uncombined

The formula, of course, involves many approximations. Some should be discussed

The amount of material is measured in terms of the nitrogen content. This is permissible on the condition that virus and inhibitor have the same nitrogen content. According to Knight (1944) this should be the case. Furthermore, the impurities (" 30 S " particles) should have the same nitrogen content. On this point no information is available at present

The particle weight is assumed proportional to the 3/2 power of the sedimentation constant. This presupposes a spherical shape of the particles and identity of partial specific volumes, about which information is insufficient

The formula given may, however, at least give an idea of the order of magnitude of the ratio mentioned

From the data in the two experiments related above the ratios 1/1 2 and 1/0 9 were computed for mixtures containing just enough inhibitor to precipitate the total virus amount added, and another experiment, not listed above (constant inhibitor versus varying virus amounts, mixtures not diluted before centrifugation) yielded the ratio 1/1 8. The values from different experiments thus appear in good agreement, indicating that about one virus particle is inhibited by one inhibitor particle. The largest proportion of virus particles incorporated in the precipitate can be calculated from the values in Fig 2. In the highest inhibitor dilution the precipitate was probably saturated with virus particles. Here about 70 virus particles were added per inhibitor particle, and as about 2/7 were fixed in the precipitate, this should contain about 20 virus particles per inhibitor particle—a rather surprising proportion of the large virus particles to the much small inhibitor particles

The observation that virus-antibodies prevent the precipitation of virus with inhibitor is in keeping with the inhibition of haemagglutination by specific antibodies. These reactions indicate that the antibody somehow blocks the chemical structures on the virus particle that combine with the inhibitor. This, of course, does not necessarily imply that the antibody and the receptor groups of the inhibitor are chemical analogues

SUMMARY

Virus, the inhibitor-destroying capacity of which has been inactivated by heat treatment, gives a precipitate with inhibitor which does not dissolve spontaneously on incubation. The composition of this precipitate varies with the proportions of the components added,

Virus antibodies prevent the precipitation when added to the virus before the inhibitor is added

This investigation was aided by a grant from the Swedish Medical Research Council

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION   THE EFFECT OF ACTIVE VIRUS, PROTEOLYTIC ENZYMES AND PERIODATE ON THE INHIBITOR

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IN the two preceding papers (Svedmyr, 1949a, 1949b) a description was given of the precipitation-dissolution reaction between purified concentrates of the PR 8 strain of influenza virus and the inhibitor present in normal allantoic fluid from chick embryos

In this paper some observations are reported regarding the effect on the inhibitor of treatment with active virus, proteolytic enzymes or periodate, as well as the action of virus and periodate upon a stable virus-inhibitor precipitate

MATERIALS AND METHODS

The virus and inhibitor preparations as well as the buffer and saline solutions used were described in the preceding publications (Svedmyr, 1949a, 1949b)

Crystalline trypsin and crystalline soybean trypsin inhibitor (Kunitz, 1946) were obtained through the courtesy of Dr Moses Kunitz, of the Rockefeller Institute for Medical Research

The trypsin preparation, containing about 50 per cent $MgSo_4$, was dissolved in saline and then carefully washed on the ultrafilter (Seibert, 1928) with several lots of saline to remove the $MgSO_4$, and then with phosphate buffer containing 100 units each of penicillin and streptomycin per ml as preservative   Finally, the stock solution was made up to contain about 10 mg trypsin per ml   The ultrafiltration was carried out at 2° C

The crystalline soybean trypsin inhibitor was kept in stock solution at a concentration of 10 mg per ml of phosphate buffer with preservatives as mentioned above

Ficin was used as a commercial preparation, also stored in a stock solution of 10 mg per ml phosphate buffer with the same preservatives

The activity of the trypsin and ficin solutions was tested on clots of human fibrin

All stock suspensions of virus, inhibitor and enzymes were stored at about 4° C

Chemically pure, crystalline $NaIO_4$ was dissolved to a concentration of m/10 in saline and kept as stock solution   Further dilutions were always made in saline and are expressed throughout in the final molarity of $NaIO_4$

A 10 per cent solution of analytically pure glucose in phosphate buffer was used as periodate-inactivator with the same preservatives as mentioned above

Haemagglutination tests were usually performed as described previously (Svedmyr, 1948a)   In several experiments, however, in addition to active virus, heat-inactivated virus (kept in the water bath at 65° C for 15 minutes) was used for the titrations of inhibitory activity   If so, the virus-inhibitor mixture was usually kept at room temperature for one hour before the addition of red cells

Some other modifications are mentioned in the text

EXPERIMENTAL

The Effect of Small Amounts of Active Virus on the Inhibitor

In a previous paper (Svedmyr, 1948b) it was shown that active virus, even in small quantities, has the capacity to gradually destroy the inhibitory activity of normal allantoic fluid   After sufficient destruction or modification of the inhibitor, haemagglutinins appear in the previously non-haemagglutinating mixture   The inhibitor-destroying capacity of the virus was eliminated by a suitable heat treatment

The following experiment was designed to study the influence of small amounts of virus on purified inhibitor concentrates, and test the results by titrations for residual inhibitory activity and by precipitation with heat-inactivated virus concentrates

Preparations of active and heat-inactivated " standard virus " (SV) and of " modified virus " (MV) were diluted 1/100 in phosphate buffer   Each dilution was mixed with inhibitor in the proportions 1 + 4, making a final concentration of 1/500 of the virus concentrate   A control with phosphate buffer substituted for the virus was included   The mixtures were incubated for 48 hours at 35° C   Samples were removed after different periods of time, heated in the 65° C water bath for 15 minutes in order to interrupt the elution process, and then stored at 4° C until titrated for residual inhibitory activity   After 48 hours of incubation a sample was also tested for capacity to precipitate with heat-inactivated virus   The results are listed in Table I

It is clear that even relatively small amounts of active virus gradually destroy both the inhibitory and the precipitating capacity of the inhibitor   The heat-inactivated virus had lost all such activity

TABLE I —*The Effect of Active and Inactive Virus Preparations in the Final Dilution 1/500 on the Inhibitor*

Virus material	Duration of incubation at 35° C	Active SV 1/500	Active MV 1/500.	Heat inactivated SV 1/500	Control without virus
Residual inhibitory activity of the inhibitor preparation incubated with virus*	0	0 75	0 98	0 67	0 82
	3 hr	0 22	0 38	_0 75	—
	18 hr	0 22		—	0 82
	48 hr	0 22†	0	0 75	0 82
Precipitation with 1/5 volume of heat-inactivated SV (test sample incubated for 48 hrs)		—	—	+	+

* Samples were removed after the indicated periods of time and heated at 65° C for 15 minutes to interrupt the process   The series with MV was then diluted in saline 1/160 and titrated directly , the other series were diluted 1/200, and all samples, except those with heat-inactivated virus, boiled before titration
† Positive agglutination before boiling

The Effect of Proteolytic Enzymes on the Inhibitor

Crystalline trypsin

Several authors have reported that crystalline trypsin destroys influenza virus receptors of red cells (Hirst, 1948), as well as the inhibitor of normal serum (Hirst, 1948, McCrea, 1948) and that of normal allantoic fluid (Hardy and Horsfall, 1948)

In a series of preliminary experiments with crude allantoic fluids it was found difficult to evaluate the results owing to the haemagglutinating effect of the trypsin itself   As previously shown by Volkert and Horsfall (1947) in experiments on pneumonia virus of mice, this difficulty can be overcome by addition of crystalline soybean trypsin inhibitor (Kunitz, 1946)   Consequently the trypsin inhibitor was used in the present experiments   In the concentrations employed it did not interfere with virus haemagglutination titres or with virus-inhibitor precipitation tests

Effect of varying concentrations —First, the influence of varying concentrations of trypsin on the inhibitor was studied   One volume of trypsin dilution was added to four volumes of the inhibitor, the final concentrations of trypsin being 2, 0 5 0 1 and 0 02 mg per ml   The mixtures were incubated at 35° C for three hours after which trypsin inhibitor was added in excess   The mixtures were then tested for capacity to precipitate with heat-inactivated virus, and titrated for residual inhibitory activity in the usual way (Table II)

Crystalline trypsin, even in a concentration of 2 mg per ml , failed to destroy the capacity of the inhibitor to precipitate with heat-inactivated virus   The inhibitory activity however, was reduced, although not completely destroyed   In this time interval about 0 1 mg of trypsin per ml   was needed for a significant effect

Control tests showed that no precipitate was formed in mixtures of trypsin (2 mg per ml) and virus without inhibitor

Effect of length of incubation —The influence of the length of incubation on the trypsin effect was tested in a similar experiment with the use of 0 1 mg of trypsin

TABLE II —*The Effect of Varying Concentrations of Crystalline Trypsin on the Inhibitor by Incubation at 35° C for 3 Hours*

Concentration of trypsin	Residual inhibitory activity* against active virus	Residual inhibitory activity* against inactivated (65° C for 15 min) virus
2 mg /ml	0 15	2 05
0 5 mg /ml	0 37	2 05
0 1 mg /ml	0 60	2 20
0 02 mg /ml	1 35	2 65
0	1 65	2 80

* Determined on a 1/150 dilution of the inhibitor material

per ml   Samples were removed after different periods of time, and the trypsin inactivated by addition of excess trypsin inhibitor (Table III)

TABLE III —*Residual Inhibitory Activity of an Inhibitor Preparation after Treatment with 0 1 mg of Crystalline Trypsin per ml for Various Periods of Time*

Duration of incubation	Residual inhibitory activity of the material treated with trypsin *	Control Trypsin already inactivated by trypsin inhibitor and added to the preparation after incubation
0	1 36	.
30 min	0 83	
3 hr	0 38+	1 36
15 hr	0 23+	

* Determined on a 1/160 dilution of the inhibitor material
+ = positive precipitation reaction with heat inactivated SV

Apparently the inhibitory activity decreased gradually, but it was not completely lost even after 15 hours   The final product also retained the capacity to precipitate with virus   The latter reaction was tested with heat-inactivated virus as well as with active virus in the cold   In both cases heavy precipitates appeared, that with active virus being dissolved in the typical way within a few minutes if kept at 35° C (Svedmyr, 1949a)

Control tests showed that increase of the concentration of trypsin to 2 mg per ml did not cause any further reduction of the inhibitory activity after 17 hours at 35° C

In one experiment the residual amount of inhibitor was assayed with two different methods   The inhibitor was treated with 0 4 mg of trypsin per ml at 35° C for 20 hours, after which an excess of trypsin inhibitor was added   The reaction mixtures were then titrated for inhibitory activity in two principally different ways, in both cases against active as well as heat-inactivated purified virus   As in other experiments a control was included—inhibitor incubated without trypsin and then mixed with trypsin already inactivated

1   The virus was serially diluted in 0 25 ml amounts of saline, 0 25 ml of the test material diluted 1/100 in saline, and finally 0 5 ml of red cell suspension were added   The mixtures of heat-inactivated virus and inhibitor were kept at room temperature for 1 hour before the addition of red cells

2 The test material was serially diluted in 0 25 ml amounts of saline, 0 25 ml containing 8 agglutinating doses of virus, and finally 0 5 ml of red cells were added Mixtures of inactive virus and inhibitor were likewise kept at 100m temperature for 1 hour before the addition of red cells

The results are listed in Table IV

TABLE IV —*Residual Inhibitory Activity* after Trypsin Treatment of the Inhibitor as Titrated with Different Methods*

Titration method	Residual inhibitory activity against active virus		Residual inhibitory activity against heat-inactivated virus	
	Material treated with trypsin	Control without trypsin treatment	Material treated with trypsin.	Control without trypsin treatment
I	0 38	1 73	2 03	2 93
II	<1 00	1 83	1·98	2 73

* Determined on a 1/100 dilution of the inhibitor material
Active and heat-inactivated SV were used as test viruses

The results show that 5–25 per cent of the inhibitory activity resisted trypsin treatment As can be seen from data in the preceding paper (Svedmyr, 1949b) a corresponding reduction of the inhibitor concentration will not have any appreciable influence upon the precipitation reaction

Ficin

An attempt was also made to destroy the precipitative capacity of the inhibitor by means of a commercial preparation of ficin It was found, however, that incubation at 35° C for 2¼ hours with 2 mg ficin per ml did not destroy the capacity of inhibitor to precipitate with heat-inactivated virus Further incubation at 35° C for two days in the presence of ficin did not change the appearance of the precipitate A control revealed that practically no precipitate was formed in mixtures of virus and ficin without inhibitor

The Effect of Periodate on the Inhibitor

Hirst (1945, 1948) described the destruction of red cell receptors and the inhibitor of normal rabbit serum by rather low concentrations of sodium periodate as opposed to other oxidants It has been shown that periodate has the same effect on virus inhibitors from other sources, such as mucins of various human origins (Anderson, Burnet, Fazekas, McCrea and Stone, 1948) and also the inhibitor of normal allantoic fluid (Hardy and Horsfall, 1948) The action of periodate has been taken as an evidence that carbohydrate is an essential part of the receptors, since it is known that periodate in biological materials chiefly attacks carbohydrates This assumption has later been very much corroborated by the isolation from red cells of very potent inhibiting substances containing a high percentage of carbohydrate (de Burgh, Yu, Howe and Bovarnick, 1948 , Woolley, 1949)

The effect of sodium periodate on the purified inhibitor concentrates from normal allantoic fluid was studied in some experiments

Effect of different concentrations

First, the effect of different concentrations of periodate on the inhibitory activity was tested Twofold serial dilutions of $NaIO_4$ were made in saline, and each dilution in an amount of 0 04 ml was mixed with 0 16 ml of inhibitor The final concentrations of periodate covered the range of M/100 to M/1600 After two hours at room temperature 0 10 ml of 10 per cent glucose was added to each sample to destroy the excess of periodate, and after dilution with 32 ml of saline, i e 200 times the original volume of inhibitor preparation, each mixture was titrated for residual inhibitory activity against both active and heat-inactivated virus For results see Table V

TABLE V —*The Effect of Different Concentrations of Periodate on the Inhibitory Activity*

Concentration of $NaIO_4$	Residual inhibitory activity against active virus	Residual inhibitory activity against inactivated (65° C for 15 min) virus
M/100	0 23	0 03
M/200	0 15	0 10
M/400	0 23	0 33
M/800	0 38	0 33
M/1600	1 66	2 51
0	1 58	2 73

Concentrations of periodate down to M/800 eliminated the inhibitory activity, the titre values shown being insignificant even against inactive virus At a dilution of M/1600, however, the periodate failed to influence the inhibitory activity

In another experiment, now with periodate in the concentration of M/150, samples were removed from the inhibitor-periodate mixture after different time intervals at room temperature and the excess periodate immediately destroyed by glucose It appeared (Table VI) that the inhibitory capacity decreased precipitately, and the final product had lost its capacity to precipitate with heat-inactivated virus

TABLE VI —*The Effect of Periodate* (M/150) *on the Inhibitor as Tested after Different Periods of Time*

Duration of incubation at room temperature	Inhibitor treated with periodate M/150		Inhibitor treated with a mixture of periodate and glucose	Control Inhibitor without periodate	
	Residual in hibitory activity	Precipitation reaction with heat inacti vated virus	Residual in hibitory activity	Residual in hibitory activity	Precipitation reaction with heat inacti vated virus
About 30 sec	0 45				
5 min	0				
2 hr	0	Negative	1 36	1 43	Positive

The titrations for inhibitory activity were performed on a 1/100 dilution of the inhibitor material

Effect on precipitation dissolution reaction —The effect of different concentrations of periodate on the precipitation-dissolution reaction of the inhibitor with active virus was tested in some experiments Various dilutions of periodate (0 01 ml) were added to 0 03 ml amounts of inhibitor The mixtures were kept for at least 2 hours at room temperature, after which 0 01 ml of 10 per cent glucose was added to each tube to destroy excess periodate The tubes were then placed in ice-water with subsequent addition of 0 01 ml of active standard virus or 0 05 ml of active modified virus Both virus preparations and pipettes were pre-chilled The appearance of precipitates was recorded, and the tubes left for at least 30 minutes in the ice-water They were then incubated at 35° C or at room temperature and the approximate dissolution times were noted (Tables VII–X)

The results reveal that with the higher concentrations of periodate, down to about м/600, the precipitation capacity of the inhibitor was destroyed, and that the lower concentrations of periodate did not influence the formation of a precipitate This is in keeping with the above-mentioned effect of various periodate concentrations on the inhibitory activity Within a rather narrow zone of periodate concentration, however, м/1440 and м/1350 respectively in two experiments, the dissolution reaction of precipitates with active SV was significantly modified

TABLE VII —*Precipitation-dissolution Reaction Between Active SV and Periodate-treated Inhibitor*

Periodate concentrations	The appearance of the precipitates after the indicated periods of time						
	Immediately at 1° C.	30–60 min at 1° C	2 min at 35° C	5 min at 35° C	15 min at 35° C	30 min at 35° C	Overnight 35° C
м/160	—	—	—	—	—	—	—
м/180	—	—	—	—	—	—	—
м/1440	+++	+++	++(+)	++(+)	++	+(+)	—
м/4320	+++	+++	++(+)	++	—	—	—
м/12,960	+++	+++	++(+)	++	—	—	—
м/38,880	+++	++(+)	++(+)	++	—	—	—
м/116,640	+++	+++	++(+)	++	—	—	—
Control inhibitor treated with a mixture of periodate and glucose	+++	+++	++(+)	++	—	—	—
Untreated inhibitor	+++	+++	++(+)	++	—	—	—

TABLE VIII —*Experiment as in Table VII   Shorter Intervals Between the Periodate Concentrations*

Periodate concentrations	The appearances of the precipitates after the indicated periods of time									
	Immediately at 1° C	30–55 min at 1° C	5 min at 35° C	10 min at 35° C	15 min at 35° C	20 min at 35° C	25 min at 35° C	30 min at 35° C	35 min at 35° C	Overnight at 35° C
м/400	—	—	—	—	—	—				—
м/600	+	+	—	—	—	—				—
м/900	+++	+++	++	—	—	—				—
м/1350	+++	+++	++	++	+(+)	+	+	(+)	—	—
м/2025	+++	+++	++	+	—	—				—
м/3040	+++	+++	++	—	—	—				—
м/4560	+++	+++	++	—	—	—				—
м/6840	+++	+++	++	—	—	—				—
Untreated inhibitor	+++	+++	++	—	—	—				—

TABLE IX —*Precipitation-dissolution Reaction Between Active MV and Periodate-Treated Inhibitor*

Periodate concentrations	The appearance of the precipitates after the indicated periods of time				
	Immediately at 1° C	30–60 min at 1° C	5 min at 35° C	10 min at 35° C	Overnight at 35° C
M/400	—	—	—	—	—
M/600	—	—	—	—	—
M/900	+++	+++	+	—	—
M/1350	+++	+++	+	—	—
M/2025	+++	+++	+	—	—
M/3040	+++	+++	+	—	—
M/4560	+++	+++	+	—	—
M/6840	+++	+++	(+)	—	—
Untreated inhibitor	+++	+++	(+)	—	—

TABLE X —*Experiment as in Table IX Shorter Intervals Between the Periodate Concentrations*

Periodate concentrations	The appearance of the precipitates after the indicated periods of time					
	Immediately at 1° C	30–60 min at 1° C	5 min at room temp	10 min at room temp	15 min at room temp	Overnight room temp
M/600	—	—	—	—	—	—
M/900	++	+	—	—	—	—
M/1080	++	+++	++	(+)	—	—
M/1200	++	+++	+	(+)	—	—
M/1350	++	+++	++	(+)	—	—
M/1620	++	+++	++	(+)	—	—
M/1800	++	+++	++	(+)	—	—
M/2025	++	+++	++	(+)	—	—
M/3040	++	+++	++	(+)	—	—
Untreated inhibitor	++	+++	+(+)	(+)	—	—

inasmuch as the reaction time was prolonged   It is striking that in one experiment there was a concentration of periodate intermediate between the " destructive " and " modifying " doses that apparently did not influence the course of the precipitation-dissolution reaction   This might, however, be an artefact, for instance the effect of accidental reduction of the periodate by threads of cotton-wool

The phenomenon of a prolonged dissolution time was not observed in precipitates containing active modified virus, although (a) rigid precautions were taken to avoid an accidental inactivation of the periodate, and (b) the intervals between the periodate concentrations were made very short

It was shown above that both active virus and suitable concentrations of periodate destroy the inhibitory activity of the inhibitor as well as their capacity to precipitate with virus   These agents were also tested for their capacity to release heat-inactivated virus particles from a fresh stable precipitate with inhibitor

In each of two tubes 0 20 ml of inhibitor was mixed with 0 05 ml of heat-inactivated standard virus   The mixtures were kept at room temperature for one hour and the precipitates were then sedimented at 3500 r p m for 20 minutes in an angle centrifuge   The heavy sediments were treated in two ways

1 One sediment was resuspended in 0 25 ml of M/100 NaIO$_4$ at room temperature   After a few minutes the precipitate appeared dissolved·   The mixture was left at 4° C overnight and then for two hours at room temperature before

the addition of 0 25 ml of a 10 per cent solution of glucose to destroy excess periodate It was then again centrifuged at 3500 r p m for 20 minutes, and the rather slight sediment resuspended in 0 25 ml phosphate buffer

2 The other sediment was resuspended in 0 25 ml of a dilution of 1/100 in phosphate buffer of active standard virus Together with a control tube with the same dilution of active virus it was then incubated at 35° C overnight The mixture still showed a large sediment which, after a final centrifugation at 3500 r p m for 20 minutes, was resuspended in 0 25 ml of phosphate buffer

The various fractions and controls, including the original heat-inactivated virus, were then titrated for haemagglutinins The results are shown in Table XI All titres are corrected for volume changes, so that the values are directly comparable

TABLE XI —*Treatment of the Virus Inhibitor Precipitate with Active Virus and Periodate*

Tested material	Haemagglutination titre
I Supernatant of the mixture before treatment	2 36
,, ,, after periodate treatment	5 44
Sediment ,, ,,	4 31
II Supernatant of the mixture before treatment	2 36
,, ,, after treatment with active virus	5 06
Sediment ,, ,, ,,	4 24
Control titre of the heat-inactivated virus	5 63
,, ,, active virus	4 16

Treatment with periodate obviously released roughly 2/3 of the haemagglutinins, and only a small fraction of the original precipitate could be recovered by centrifugation Active virus left the bulk of the precipitate unchanged, and only about 1/4 of the haemagglutinins were released It may be mentioned, however, that in another experiment with a precipitate several weeks old much less haemagglutinin was released whether periodate or active virus were used On the other hand, in a similar control experiment a fresh precipitate of inactive virus and inhibitor was not visibly altered when incubated at 35° C for one week in a suspension containing active virus

It may be mentioned that previous trypsin treatment of the inhibitor component of the precipitate did not influence the capacity of periodate to dissolve the precipitate Thus the floccules formed in the experiment listed in Table II were dissolved in a few minutes after addition of $NaIO_4$ in a final concentration of M/150 The same concentration of periodate destroyed the capacity of a trypsin-treated inhibitor preparation (2 mg trypsin per ml 35° C overnight) to precipitate with heat-inactivated virus

DISCUSSION

Hirst (1942) suggested that the adsorption and elution of influenza virus to red cells was due to the interaction between an enzyme incorporated in the virus particle and its substrate, the receptor, on the red cell surface This hypothesis was later adapted for the similar adsorption-elution of influenza virus to the cells

in the excised mouse lung (Hirst, 1943)   The inhibiting effect of normal serum (Francis, 1947 , Hirst, 1948 , Anderson, 1948) and of mucins of various origins (Anderson et al , 1948), as well as the destruction of this effect on incubation with virus, has been explained on the same basis

The evidence for this enzyme theory is twofold , the reaction can be repeated many times with the same aliquot of virus without reduction of virus activity , furthermore, the elution process may be prevented by previous heating of the virus to 56° C for 30 minutes

As yet little is known about the chemical structure of the receptors of different origins, and nothing about the change in this configuration following the action of the presumptive virus enzyme   A considerable amount of data has accumulated that seems to indicate that the different receptors are mucoproteins, as indicated by the sensitivity to periodate and trypsin (Hirst, 1945, 1948 , Anderson et al , 1948 , Hardy and Horsfall, 1948), by the high inhibitory activity of crude mucinous suspensions, and of purified mucins of different human origins (Anderson et al , 1948), and above all by the preparation from red cells of very active inhibitors containing a large percentage of carbohydrate (de Burgh et al , 1948 , Woolley, 1949)

The biological implication of the presumptive viral enzyme has been postulated first by Hirst (1943) and later by Burnet (1948a), who suggested that the initial stage of influenza virus infection was the adsorption of virus to receptors on the cell surface   After enzymatic modification of the receptor, the virus was then ready for the next step in the infection   The significance of an adsorption of virus to the cell receptors seems established by the prophylactic effect of the " receptor-destroying enzyme " against influenza virus infection in eggs and mice (Stone, 1948a, b)   For the next step in the infection Fazekas and Graham (1949), however, very recently formulated a new hypothesis   Fazekas (1949) showed that moderate doses of periodate modified the receptors of red cells and of cells lining the allantoic cavity of chick embryos   He stated that virus adsorbed to modified cells could not elute, and as he was also able to show that virus could infect the allantoic cavity even if the receptors were modified, he concluded that the viral enzyme action on the receptors is not an obligate phase in the initial stages of influenza virus infection

The inhibitor of the influenza virus haemagglutination present in normal allantoic fluid has been described by the author in previous publications   The bulk of the results are in keeping with the observations regarding other virus receptors as summarized above

This inhibitor has been shown to be a particulate component that appears in the allantoic fluid in increasing amounts after the 7th day of incubation   It seems, thus, that it is a product of cell activity, probably released cell receptors

It is of particular interest that the interaction between virus and inhibitor manifests itself macroscopically as a precipitation reaction, the combination phase, followed by dissolution of the precipitate, the elution phase   It is remarkable that the precipitate, as studied in the stable form with inactive virus, varies in composition within wide limits, depending on the proportions of added ingredients

The observation that the inhibitor-destroying capacity follows the other activities of the virus particle through a two-cycle differential centrifugation strongly indicates that it is connected with the virus particle itself   It was shown

that the capacities of the virus particle (*a*) to infect, (*b*) to destroy the inhibitory activity and (*c*) to combine with the inhibitor or to haemagglutinate may be successively destroyed by suitable treatment

Trypsin digestion of the inhibitor obviously causes an appreciable reduction in inhibitory activity Contrary to the previously cited observations, it was, however, found impossible to destroy completely the inhibitory activity, whether by increase of enzyme concentration or by prolongation of the incubation The reaction rate was relatively rapid at the start and then progressively decreased This coincides with the assumption that the inhibitor substance is enzymatically broken down Also there is nothing to indicate that the decrease of inhibitory activity is caused only by a block of the reactive groups through a combination of inhibitor and trypsin

As an explanation for this phenomenon two possibilities immediately suggest themselves

(*a*) Provided all receptor groups have a similar chemical structure, the trypsin digestion apparently causes a decrease only of their reactivity without complete loss of their capacity to combine with virus Evidence that there is such a modification of the receptor groups may be found in the observation that the trypsin effect seems to be greater if measured with active virus than with inactive (Tables II and IV), which may indicate more rapid destruction of the inhibitor by the viral enzyme following trypsin treatment

(*b*) Another possibility is that the inhibitor effect refers to two or more chemically different substances with different resistance against trypsin digestion In this connection it may be pointed out that the inhibitor preparations used contained several components that could be differentiated by sedimentation analysis Although previous observations indicate that the inhibitory activity should be associated only with the so-called 200S component, it seems not unlikely that these large particles might contain chemically different receptor groups Further investigation is obviously needed

Treatment with periodate in concentrations above M/800 completely destroyed the combining capacity of the inhibitor Compared with the limited effect of trypsin, this indicates that carbohydrate is more essential than the protein part of the receptor complex

The observations of Burnet (1948*b*) and Fazekas (1949) that treatment with moderate concentrations of periodate modifies the receptor, rendering it non-digestible by the viral enzyme, have been partially confirmed Treatment of the inhibitor with periodate within a low, very narrow concentration zone of periodate prolonged the time needed for dissolution of a precipitate of this modified inhibitor and active standard virus The dissolution reaction was, however, never completely prevented, the reaction time was only prolonged As the precipitation-dissolution reaction in all other cases seems to reflect the course of the combination-elution reaction between virus and inhibitor, it may be assumed that these modifying doses of periodate, somehow, altered the inhibitor particles, rendering them less sensible to the presumptive enzyme of the standard virus

Actually, Fazekas (1949) showed only that no significant elution of virus took place in a period of time that exceeded only slightly the time needed for elution in the controls The above data suggest that a much longer incubation might be needed for elution of virus from modified receptors If, thus, the viral enzyme action is only retarded by modification of receptors, then the observation

of infection through modified receptors does not prove the dispensability of the viral enzyme action on the receptors for the initiation of influenza virus infection

It is tempting to speculate about the implications of the fact that the periodate almost completely dissolved the precipitate while the active virus had only a limited effect

If we consider the special relationships in the virus-inhibitor complex, it is easy to visualize how the small periodate molecules can gain access to the reactive sites of the inhibitor, whereas a similar effect of the large virus particle seems to require dissociation of the virus-inhibitor bonds   Hirst (1949), on the basis of such considerations, arrived at the conclusion that the combination of inactive virus and inhibitor represents an association-dissociation equilibrium   The fact that in the experiments described above only a minor part of the virus was released suggests that certain of the bonds between virus particles and inhibitor might be stronger than others   At any rate a complete dissociability of the inhibitor-inactive virus complex seems unlikely

SUMMARY

Observations are reported regarding the modified course of the interaction between purified concentrates of influenza virus and the inhibitor of normal allantoic fluid by suitable treatment of the inhibitor

Treatment of the inhibitor even with small amounts of active virus gradually destroys both the haemagglutination inhibitory capacity and the capacity to precipitate with high concentrations of active or inactive viruses

Sodium periodate in rather low concentrations also destroys the inhibitory and precipitating capacities of the inhibitor particles   In a lower, very narrow concentration zone of periodate, however, the precipitate with active standard virus was formed as usual, but its dissolution took a longer time

Proteolytic enzymes, as crystalline trypsin and ficin, on the other hand, even in relatively high concentrations fail to abolish the capacity of the inhibitor particles to precipitate with active or inactive viruses, the precipitate with active virus redissolving in a typical way when incubated   Correspondingly, trypsin diminishes the haemagglutination inhibitory capacity only to 5–25 per cent of the original

A stable precipitate of inactive virus and inhibitor could be partly dissolved by treatment with low concentrations of active virus or periodate   Periodate released about 2/3 of the haemagglutinins from a fresh complex, only a small part of the precipitate remaining   Active virus left the precipitate at roughly the same level, only about 1/4 of the haemagglutinins being released

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EXPERIMENTAL LIVER CIRRHOSIS IN RATS PRODUCED BY PROLONGED SUBCUTANEOUS ADMINISTRATION OF SOLUTIONS OF TANNIC ACID

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IT has been shown that a cirrhosis, resembling human cirrhosis of the Laennec type, can be produced in experimental animals by abnormal diets (Glynn, Himsworth and Lindan, 1948 , Wahi, 1949) On the other hand, Cameron and Karunaratne (1936) and Steinberg and Martin (1946) have shown that the experimental cirrhosis of the liver may, in its reversible stage, end in complete anatomical recovery These facts gave rise to the hope that appropriate dietetic treatment might be successful in human cirrhosis It is now well known that the ingestion of a suitable diet and lipotropic substances frequently exerts a favourable effect on human cirrhosis, which can be confirmed histologically Unfortunately, the same therapy may fail completely in other, occasionally not advanced cases These facts indicate, in our opinion, that cirrhosis of the liver is not invariably a deficiency disease, but that other factors can play a part in its aetiology

One of us (B K) assumed as early as 1942, independently of English (Cameron, Milton and Allen, 1943) and American authors (Wells, Humphrey and Coll, 1942), on the basis of the changes found in the liver of persons who had suffered burns and been treated with tannic acid, that tannic acid had a hepatotoxic effect He administered tannic acid solution parenterally to experimental animals and found central acinar necrosis in the livers of those animals which died after 24 to 26 hours Vascular phenomena simulating the pattern of a serous hepatitis may be observed before the development of the parenchymal lesion (Korpássy, 1949)

The hepatotoxic effect of tannic acid having been proved, the question of what effect the protracted treatment with sublethal doses of tannic acid would have on the liver arose, knowing that acute poisoning leads to central acinar necrosis

EXPERIMENTAL METHODS

Twenty white rats aged about 3 months were used Their average weight was 112 g , varying between 90 g and 130 g Male and female animals were used , they were all of the same strain, the origin of which was not known The animals received a mixed diet of waste food from the hospitals, consisting mainly of milk-bread, potatoes, farinaceous food and sometimes fresh curd No accurate diet could be maintained, so white rats, untreated or used for other experiments, were kept on the same diet and served as controls Changes in the liver parenchyma could not be found either in the untreated rats or in those employed in other experiments, whether they died or were killed, though several animals were rather old at the time of the examination The untreated animals grew and reproduced normally on this diet

Treatment

All rats were given 10 mg of tannic acid on days 1, 3, 5 and 7 of the experiment On days 9, 10, 15, 17, 19, 29, 51 and 53 the dose given was 20 mg , on days 60, 61, 93, 102, 109, 114 and 116 the dose was 30 mg , on days 65, 69, 84, 118, 123, 126, 132, 137 and 142 the dose was 40 mg , on days 147, 153, 159, 165, 172 and 194 the dose was 50 mg , on days 200 and 208 the dose was 60 mg , and on day 215, 70 mg were given The tannic acid was administered subcutaneously as a 1 per cent solution for the first 10 doses, and thereafter as a 2 per cent solution Four animals died on day 19, 2 on day 29, 2 on day 31, 1 on day 34, 2 on day 39, and 1 on each of days 70, 71, 85, 94, 217 and 232 On each of days 69, 141 and 195 a rat was killed The average weight fell from 112 g to 96 g in 19 days On day 39 the average weight was 120 g , on day 69 it was 152 g , on day 102 it was 175 g , on day 137 it was 187 g , on day 166 it was 176 g , on day 190 it was 180 g , and on day 210 it was 195 g

Necrosis occurred at the site of the subcutaneous injections after the 20th day, and ulcers 2 to 3 cm diameter were left after separation of the necrotic tissue All the ulcers healed without special treatment, and no inflammation of their margins or in the deep layers was observed In no case could the death of the animal be attributed to these local processes

All the animals were dissected as soon as possible The organs of 18 rats were examined , 2 rats which died on days 19 and 39 had been devoured by the others

Gross and microscopic changes in the liver

In order to obtain a clear picture of the changes due to the prolonged treatment the animals were grouped according to the time of their death The first group contained the animals which died up to the 39th day , the second group contained those which died between days 69 and 94 , the animals in the third group survived 100 days

19

Group I (9 rats) —The liver was slightly swollen, with a smooth surface, and had the structure of nutmeg liver in miniature   Histological examination showed a varying degree of central breakdown of the lobules   The débris had for the most part been removed and its place taken by red cells, resulting from dilatation or rupture of the central vein and neighbouring sinusoids   In the livers of those animals that died between 20 and 26 hours after the last treatment, karyorrhexis of the central cells could be seen   In the peripheral zone of the lobules the normal radial structure had disappeared   The liver cells were generally enlarged, nevertheless they displayed striking variations in size, and there were numerous cells in which both the cytoplasm and the nucleus were enormously enlarged (Fig 1)   In addition, double nuclei and cell divisions could be seen   The nuclear staining was uneven , liver cells with pale vesicular-nuclei were interspersed with others whose nuclei were hyperchromatic and pyknotic Not infrequently round acidophil inclusion bodies could be seen in the vesicular nuclei   Large vacuoles appeared in the cytoplasm of some cells, but fat in small droplets could rarely be demonstrated in the sections with Sudan III   In some cases where there had been central haemorrhagic necrosis, accumulations of haemosiderin were present in the parenchymal cells   In other cases the cells contained light yellow granular bile pigment

There were other changes deserving attention   As early as the 19th day there were in the sections small or medium-sized elongated cells with a bright staining nucleus, and these cells were more numerous in the livers of the animals which died later   These cells were arranged in short or long columns and strands between the remaining cords of liver cells   Sometimes they were situated on one side of the dilated central vein like a crescent or cap and they seemed to invade the liver tissue from here   As a rule, the number of these cells was increased in the periportal tissue also

The behaviour of the reticulum as shown by Gömöri's impregnation was rather characteristic   Initially, the fibrous meshwork was collapsed in the region of the central acinar necrosis and broken to pieces on the periphery (Fig 4) Later on, after the 30th day, the primary thickening of the reticulum fibres could be seen, mainly around the central veins, and to a lesser degree around the interlobular vessels (Fig 5)

Group 2 —In 2 of the 5 rats belonging to this group (they died on days 71 and 85 respectively) the gross changes were well marked   The liver was enlarged

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EXPLANATION OF PLATES

Fig 1   Rat T/11   Treated with 180 mg tannic acid administered in 11 doses   Died on 29th day   Haematoxylin and eosin   × 210

Fig 2 —Rat T/6   18 injections, total 400 mg tannic acid   Died on 85th day   Haematoxylin and eosin   × 360

Fig 3 —Rat T/18   28 injections, total 750 mg tannic acid   Killed on 141st day Haematoxylin and eosin   × 54

Fig 4 —Rat T/11   10 injections, total 160 mg tannic acid   Died on 19th day   Gömöri's reticulum stain   × 57

Fig 5 —Rat T/13   Treated with 180 mg tannic acid administered in 11 doses   Died on 31st day   Gömöri's reticulum stain.   × 57

Fig 6 —Rat T/5   16 injections, total 320 mg tannic acid   Died on 69th day   Gömöri's' reticulum stain   × 57

Fig 7 —Rat T/18   28 injections, total 750 mg tannic acid   Killed on 141st day   Gömöri's impregnation   × 57

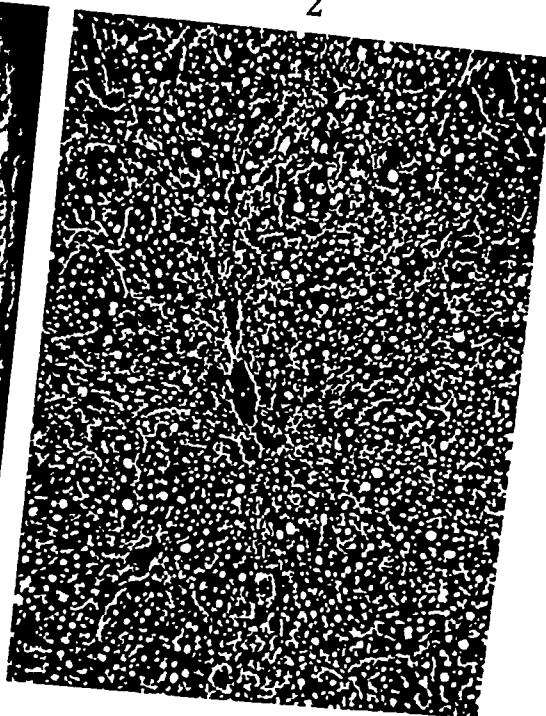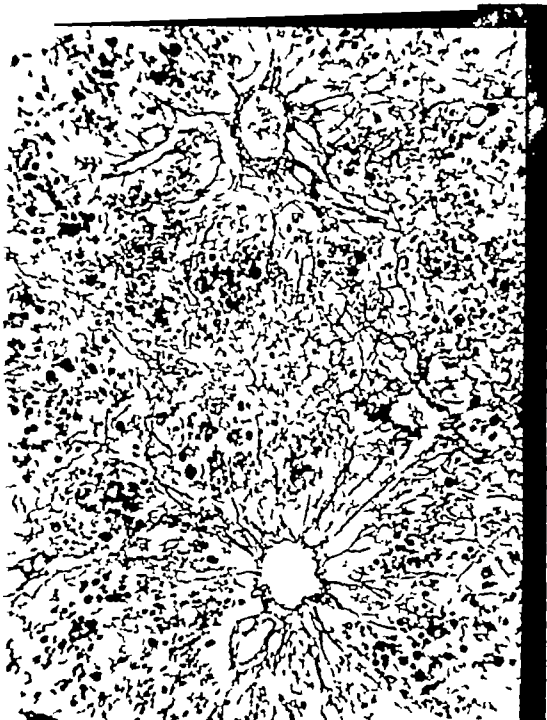Fig 8 —Rat T/19   38 injections, total 1280 mg tannic acid   Died on 217th day

1

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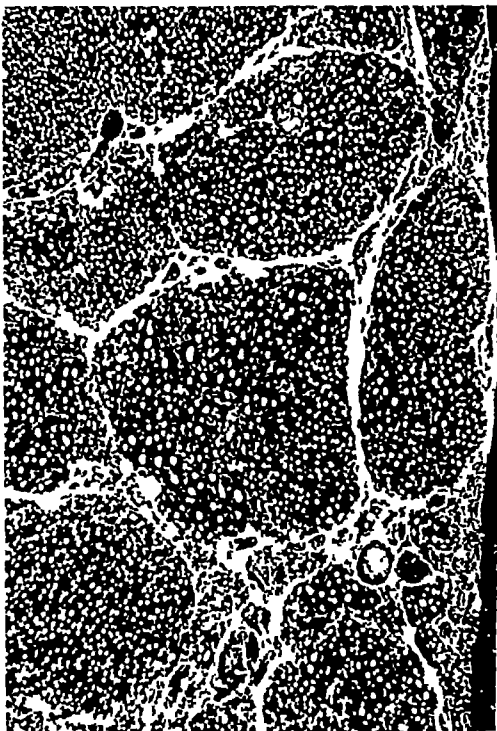
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Korpassy and Kovacs
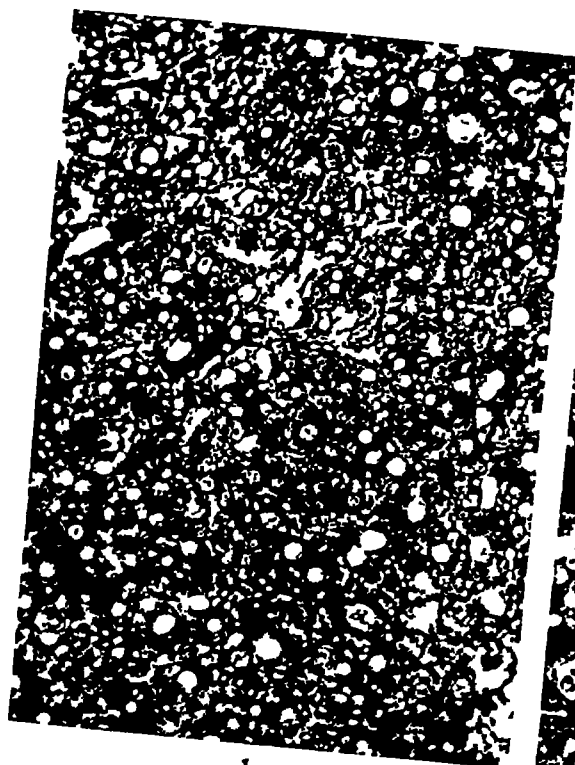
and firm with its surface finely and evenly granular, especially when seen with a lens The breakdown of the architecture of the liver was conspicuous in all cases and the polymorphism of the liver cells was more marked than in Group 1 The elongated cells mentioned under Group I were present in enormous numbers and not infrequently they formed thick strands extending from the centre of one acinus into another In other places these cells were oval or flattened and here and there formed wreath-like structures

The bile ducts were invariably increased in numbers, and groups of 8 or 10 were sometimes found between the lobules or in the interior of the disintegrated lobules These bile ducts, some of which had no apparent lumen (Fig 2), were often surrounded by the elongated cells previously mentioned The reticulum fibres were likewise increased in numbers and thickened The gradual transformation of the architecture of the liver could be followed well *in sections stained by Gömöri's impregnation*, a method which demonstrates the liver cells in addition to the reticulum The thickened and more numerous reticulum fibres, forming strands of varying thickness, delineated and clearly indicated the irregular disintegrated lobules, and here and there surrounded groups of surviving liver cells (Fig 6)

Sections of the liver of the rat which died on the 85th day, stained by van Gieson's method, showed collagen fibres in some of the thickened strands of connective tissue Traces of fat and glycogen could also be found

Group 3 (4 animals) —In 3 animals (treated for 141, 195 and 217 days respectively) it was obvious on external examination of the liver that it was diseased The surface was finely and evenly granular, the greyish-brown nodules about the size of millet seeds were sharply defined and were separated by dark coloured furrows (Fig 8) The cut surface showed that the granularity extended uniformly throughout the organ

The histological picture was very similar to that of human cirrhosis of the Laennec type The nodules corresponded to groups of liver cells surrounded by bands of connective tissue of varying thickness These nodules are pseudo-lobules , the hepatic cells are not arranged in any definite fashion, and there is no central vein or the vein of the lobule is situated at the periphery (Fig 3) The cells constituting these pseudo-lobules were markedly polymorphic, but the endothelium lining the rather wide sinusoids appeared normal The pseudo-lobules had a delicate reticulum meshwork, in sharp contrast to the coarser surrounding fibres (Fig 7) The small elongated cells were increased in number only in the environment of the liver cell groups In two cases the proliferation of the bile ducts resulted in structures resembling adenomas

In the liver of the rat which survived all the others, moderate fibrosis was present, but the characteristic distortion of the architecture was absent

RESULTS

Histological examination has shown that the protracted *parenteral* administration of tannic acid results in a gradually progressive destruction of the liver parenchyma and transformation of its architecture In the early stages breakdown and subsequent regeneration of the liver cells are the most striking features , direct and indirect cell divisions may be seen in the marginal cells of the lobules, while other nuclei are, owing to their inner division, enormously enlarged Changes

in the reticulum seem to be a good measure of the degree of transformation of the
liver architecture   During the first week or two the reticulum fibres are moder-
ately thickened and increased in numbers, particularly around the central
veins   By the 70th day the increase and thickening of the reticulum is con-
siderable and the broken down liver parenchyma is interwoven by strands of
reticulum of varying thickness which split off pieces of varying size and shape
from the lobules   At the same time the proliferation of the bile ducts sets in
This stage corresponds, in our opinion, to an early cirrhosis or precirrhosis

In rats which survived 100 days of treatment the architecture of the liver
was already distorted or completely transformed   The remnants of the paren-
chyma form round nodules of various sizes surrounded by a connective tissue
meshwork containing collagen   The proliferation of the bile ducts simulates
adenoma at many places   The changes seen in the liver of the rat killed on day
141 correspond to a diffuse fibrosis indistinguishable from the classical pattern
of portal (Laennec) cirrhosis

We attribute a particular role in this transformation of the liver structure
(tannic acid cirrhosis) to the small elongated cells which were observed initially
in small, later in gradually increasing, numbers   These cells are, in our opinion,
of reticulo-endothelial origin and they seem to play some part other than reticulum
formation   Our serial sections seem to indicate that these small immature cells
may develop into bile ducts (i e epithelial cells of the bile ducts) and also occa-
sionally those small liver cells which could be found in their environment   The
presumption may be warranted that this strong hepatotoxic compound, or perhaps
the repeated destruction of the parenchyma, has the sequel that the remaining
reticulo-endothelium takes on the pluripotential properties of the embryonal
mesenchyma

DISCUSSION

There are numerous papers in the literature dealing with the experimental
production of cirrhosis   Besides the purely dietetic procedures mentioned in
the introduction to this paper, there are many organic and inorganic substances—
drugs, tar-like materials, proteins and their decomposition products, bacterial
toxins, etc —which produce liver lesions when administered to experimental
animals (Moon, 1934)   The results of these experiments are not quoted here
as the lesions reported are quite unlike the classical human cirrhosis of Laennec
type

Mallory (1925, 1933 , Mallory and Parker, 1931) was engaged in the problem
of the aetiology of cirrhosis of the liver for several decades   Having ruled out
ethyl alcohol as exclusively responsible, as other authors such as Friedenwald
(1905) had also done, he performed extensive experiments to examine the effects
of those substances which might contaminate alcoholic beverages   He examined
more than 30 substances, including copper, lead, aluminium, antimony, zinc,
arsenic, sulphates, furfurol, turpentine, and creosote   Ultimately, on the basis
of his experiments in 1933 with phosphorus, he claimed that Laennec's cirrhosis
was due to the contamination of beverages with phosphorus   However, the
experimental phosphorus cirrhosis produced by Mallory bears little relation to
human cirrhosis of the Laennec type

There are, in fact, not many chemical substances capable of producing cirrhosis
in a strict sense in animals   Beattie and Dickson (1948) hold that manganese

and its salts belong to the small group of substances which produce a hepatic lesion resembling human cirrhosis (Findlay, 1924, Hurst and Hurst, 1928) Many authors have of recent years been concerned with the effect of carbon tetrachloride (Cameron and Karunaratne, 1936, Sundareson, 1942, Ungar, 1945, and others) Ashburn, Endicott, Daft and Lillie (1947), unlike the other authors, contend that the first alteration due to carbon tetrachloride is proliferation of the connective tissue around the central veins Since the discovery of Kinoshita (1937) attention has been directed to the cirrhogenic and carcinogenic effect of the azo dyes, especially butter yellow The combination of different agents has proved more effective than the application of the individual substances (Moon, 1934)

Having compared the histological changes resulting from the protracted subcutaneous application of tannic acid, butter yellow, and other substances it appeared to us that the cirrhoses produced by carbon tetrachloride and butter yellow bore the greatest resemblance to tannic acid cirrhosis The cirrhoses due to tannic acid and carbon tetrachloride may have a similar histogenesis, for in both the transformation of the architecture of the liver seems to start from the centre of the lobules Nevertheless, wherever the process which results in diffuse fibrosis may begin, the final pattern cannot be distinguished from that of classical portal cirrhosis, as has recently been stressed with regard to dietetic cirrhosis (Glynn, Himsworth and Lindan, 1948)

Finally, the question arises whether the various substances which can produce cirrhosis in experimental animals play any part in the genesis of human cirrhosis The majority probably play no part, but no definite opinion as to the aetiological role of tannic acid can be formed for the time being Further investigations are being carried out in order to answer, amongst others, the following questions (1) Have the skin necroses and ulcerations, which inevitably occur with the subcutaneous applications of tannic acid, any influence on the development of the cirrhosis ? (2) Is the effect of oral administration identical with that of injection ? In any case, we feel that the recognition of the fact that tannic acid can produce cirrhosis in experimental animals opens up a further line of investigation into this disputed problem

SUMMARY

1 The authors have produced diffuse nodular fibrosis of the liver in rats by the prolonged subcutaneous administration of tannic acid at various intervals

2 As a result of the repeated administration of tannic acid, a gradual breakdown of the parenchyma and a progressive transformation of the liver architecture takes place The first sign of this process is an increase of the reticulum around the central veins, corresponding with the central acinar destruction of the parenchyma

3 By the 70th day of treatment fibre strands connecting adjacent lobules have been developed, and at the same time the regenerative proliferation of the bile ducts becomes conspicuous (precirrhosis)

4 The picture observed on the 141st day was indistinguishable from classical portal cirrhosis (Laennec type)

5 All rats dying between days 69 and 94 of the treatment developed changes corresponding to precirrhosis, whereas in 3 of the animals surviving 100 days a diffuse nodular cirrhosis was apparent on gross examination

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THE KAOLIN-ADSORPTION METHOD FOR THE QUANTITATIVE ASSAY OF URINARY GONADOTROPHINS

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THE concentration of gonadotrophic substances in normal male and female urine is so low, that for their assay concentrated extracts must be prepared, freed from toxic substances and other hormones, especially oestrogens and androgens   It has been found that the gonadotrophic substances of menopausal or castrate urine, and probably of normal urine as well, are more susceptible to any drastic extraction process than those of pregnancy urine   For example, 60 per cent acetone containing 4 per cent ammonia destroys the menopausal hormone but not the pregnancy hormone (Evans and Simpson, 1934)   Therefore, mild methods of extraction must be used

Crude extracts may be prepared by the original alcohol-precipitation method of Zondek (1930) as modified by Leonard and Smith (1934), Frank, Salmon and Friedman (1934), Heller and Heller (1939), Heller and Chandler (1942), Frank and Berman (1939) and Frank (1939), Varney and Koch (1942) and many others, by the tannic precipitation method elaborated by Levin and Tyndale (1936, 1937) or by the method involving adsorption on benzoic acid originated by

Katzman and Doisy (1932, 1934) The extracts prepared by these methods cannot be injected into an animal in the doses equivalent to large amounts of urine, as is necessary with normal male or female urine, unless their toxicity is reduced Some detoxicating procedures, such as dialysis, decrease not only the toxicity of the extract but also the activity The ultrafiltration method (Jungck, Maddock and Heller, 1947) seems to be even less efficient quantitatively than the alcohol-precipitation-dialysis procedure

Scott (1940), for purposes of biological pregnancy diagnosis, adsorbed the chorionic gonadotrophin in diluted urine on kaolin Water soluble toxic substances, such as excess of inorganic salts (Main, 1939), remained in the discarded supernatant The hormone was extracted from the kaolin precipitate with alkali and after acidification injected into rabbits, mice, or toads This method has been adopted to the assay of gonadotrophins in normal male and female urine The proteins in the alkaline extract were freed from steroids by precipitation with acetone after acidification The final, relatively non-toxic, crude concentrate of any urine can be quantitatively bioassayed, the yields being apparently sufficient to make further purification, isolation and standardization more practicable

METHODS

Extraction of urinary gonadotrophins

There are three steps in the method used adsorption of the hormones from diluted urine with kaolin at pH 4 0, extraction of the hormones from the kaolin by N/10 NaOH and precipitation of the hormones by cold acetone, the precipitate being washed with ether

In preliminary work to identify the gonadotrophic effects, 1 litre of urine was taken from a fresh 24-hour (or on some occasions 48-hour) specimen, diluted with an equal volume of distilled water and acidified with 20 per cent HCl to pH 4 0 (glass electrode) Well-shaken 20 per cent aqueous suspension of kaolin B D H washed with acids (2 5 g of kaolin per 100 c c of native urine) was added to the urine, the mixture being shaken vigorously on the shaking-machine for 1 hour and then allowed to stand for a few hours When the kaolin had settled down, the clear supernatant was syphoned off and the kaolin suspension transferred to round-bottomed centrifuge tubes and centrifuged at 2500–3000 r p m for 10 min The supernatant fluid was poured off and the kaolin-precipitate carefully ground up with N/10 NaOH (6 c c per 100 c c of original urine) until the suspension became homogeneous and free from all lumps The alkaline kaolin-suspension was then centrifuged for 10 min at 3000 r p m and the supernatant fluid (about 70 c c in volume), containing gonadotrophins, was collected and acidified to approximately pH 5 5–6 0 with 20 per cent HCl, drop by drop Five volumes (about 350 c c) of cold acetone were added to the extract, shaken vigorously and centrifuged for 10 min at 3000 r p m , the acetone being decanted and the precipitate washed with ether (pro narcosi) and dried for at least 1 hour, in a vacuum desiccator in the presence of P_2O_5 and H_2SO_4 To remove the last traces of the solvents the precipitate was dried for 24 to 48 hours in a vacuum desiccator yielding " crude gonadotrophins," a brown-coloured amorphous solid weighing up to 400 mg per litre of urine For quantitative assay the total 24 hour urinary specimen was used and extracted likewise

For the injection the fresh precipitate or "crude gonadotrophins" were suspended or dissolved in 5 c c or more 0 9 per cent saline, 1 c c of the extract (adjusted if necessary to approximately pH 6 0–6 5) being equivalent to 200 c c or less of urine   Usually both the preparation and the first injection of the fresh extract were accomplished on the same or on the next day

Identification of gonadotrophins

Only normal immature 21-day old albino rats from Wistar stock, fed on an Aberdeen standard diet, were used   For the purposes of the experiments, no attention was paid to the litters   The extracts were tested for their primary and secondary effects on the weight of the ovaries, testes, uterus, prostate and seminal vesicles   In each animal the organs were examined macroscopically, and usually microscopically and vaginal smears were carried out in all female rats   The extracts were injected subcutaneously into groups of five rats in three equal doses spread over three days, unless the contrary is stated   The vaginal smears were examined five times within 72 to 120 hours after the first injection   On some occasions at this time female rats were vitally stained with 2 c c 2 per cent trypan blue injected subcutaneously

Male rats were killed 72 hours and female 120 hours after the first injection, the reproductive organs being fixed in Bouin's fluid overnight and then dissected, drained on filter paper and weighed on a torsion balance   Before weighing, the ovaries and testes were decapsulated and the uteri were cut off near the cervix and the oviduct, and the intra-uterine fluid if present squeezed out   The lobes of the prostate were separated from the urethra and the whole prostate was weighed, the seminal vesicles being dissected and weighed separately   At necropsy the weight of the control and treated animals was from 35 to 50 g For the average weight of dissected organs see Tables I and II

The morphological effects of the concentrates of urine were compared by complete sections of both the experimental and control gonads   The other organs, fixed already in Bouin's fluid were also cut in serial paraffin sections The haemalum-eosin method and azocarmine-aniline blue and orange-G method were used for general microscopic examination   The contrast staining for study the ovaries of the rats vitally stained with trypan blue was carried out with an aqueous solution of safranin

Quantitative bioassay

Mouse uterine technique (Levin and Tyndale, 1936, 1937) was adopted Normal immature 21-day old female mice weighing from 7 to 10 g were used The extracts were injected subcutaneously once daily up to 0 6 c c at 24 hour intervals on three consecutive days   The animals were killed 72 hours after the first injection, the uterus being removed, dissected, freed from intra-uterine fluid by pressure between pieces of filter paper, and while fresh weighed on a torsion balance   At necropsy the ovaries were examined and on some occasions also dissected and weighed   Two to five mice were used on each dosage level The weight of killed animals was from 8 to 12 g   For the average weight of dissected organs see Tables III and IV

The mouse (uterus) unit was defined as the amount of activity to produce 72 hours after the first injection a 100 to 150 per cent increase over the controls in the average uterine weight of those treated with extract.

Recovery experiments

These experiments tested the recovery from 0 9 per cent saline and fresh urine of a normal girl of added " crude gonadotrophins " from the urine of post-menopausal women   The final dried postmenopausal precipitate weighing 382 42 mg was dissolved in 18 c c of distilled water and divided into three portions each of 6 c c in volume   One portion served as a standard preparation the second was dissolved in 100 c c of 0 9 per cent saline and the third in 100 c c of urine, in which the actual gonadotrophin content was determined   Both the saline and urinary specimens with the postmenopausal gonadotrophins added were put through the kaolin adsorption procedure and then the reprecipitated gonadotrophins dissolved up to the original volume of 6 c c of 0 9 per cent saline and quantitatively assayed in comparison with the original gonadotrophin content of both the postmenopausal and normal girl urine

RESULTS

Effect of urinary gonadotrophins on immature rats

The average results of these experiments are given in Tables I and II   The microscopic changes are shown in Fig 1 to 11   The figures representing the average of the number of rats and their responses on each dose level shows the reliability of the results obtained with the kaolin-adsorption method as compared with other methods

TABLE I —*Response of Female Reproductive Organs in Normal Immature Rats to Concentrates of Urinary Gonadotrophins*

Subject, Sex, Age (M) = male (F) = female	No rats	Treatment			Necropsy Mean weight of			Increase over controls	
		Total dose c c	Equivalent of urine c c	Vaginal smears* +,±,−	Body g	Uterus mg	Ovaries mg	Uterus per cent	Ovaries per cent
	20	—	—	—	48 7	18 8	10 6	—	—
W (F) 60	5	0 5	50	±	48 0	57 6	20 0	205 3	88 6
	5	1 5	150	+	49 4	73 0	35 0	288 2	230 1
	5	2 0	200	+	36 2	78 6	42 2	318 0	279 2
S (F) 72	5	1 65(1)	200	+	48 0	84 0	44 6	346 8	320 7
G (F) 56	5	2 0	200	+	47 8	80 0	40 6	325 5	283 0
M (F) 73	1	1 0(2)	200	+	46 0	80 0	40 0	325 5	277 3
	1	1 5(2)	300	+	40 0	85 0	48 0	352 1	352 8
	5	2 0(2)	400	+	36 0	83 3	50 0	343 0	371 7
L (M) 17	5	0 5	50	—	47 4	18 6	10 6	—	—
	5	1 0	100	—	46 2	22 4	11 0	19 1	3 7
	4	1 5	150	±	40 2	26 7	12 8	42 0	20 7
	5	2 0	200	+	38 6	47 8	17 6	154 2	66 0
A (F) 22(3)	5	2 0	200	±	45 8	34 8	23 0	85 1	116 9
B (F) 20(3)	5	2 0	200	±	45 8	28 0	19 2	48 9	81 1
R (F) 16(3)	5	2 0	200	±	45 4	42 8	22 4	127 4	111 3

(1) 24 hour output = 825 c c
(2) Precipitate diluted in 5 c c 0 9 per cent saline   Five injections within 3 days   Single results for 1 0 and 1 5 c c
(3) Nullipara with regular menstrual cycle   No samples collected on the days 12 and 13 and 20 and 21 after the beginning of menses in order to avoid any interference of increased gonadotrophin content   (d'Amour, Funk and Liverman, 1939 , Levin, 1941)
* Cornified cells (only) in none (−), in some (±), in all (−) treated rats

TABLE II —*Response of Male Reproductive Organs in Normal Immature Rats to Concentrates of Urinary Gonadotrophins*

Subject, Sex, Age (M) = male (F) = female	No rats	Treatment		Necropsy Mean weight of				Increase over controls.		
		Total dose c c	Equivalent of urine c c	Body g	Testes mg	Prostate mg	Seminal vesicles mg	Testes per cent.	Prostate per cent	Seminal vesicles per cent
	10	—	—	44 8	156 0	29 8	7 2	—	—	—
R (F) 8(¹)	5	0 5	50	37 8	160 8	33 8	9 0	3 0	13 4	25 0
	5	1 0	100	43 8	216 6	51 8	10 4	39 0	73 8	44 4
	5	2 0	200	47 0	310 4	85 4	25 0	99 0	186 5	233 3
T (F) 70	4	2 0	200	42 0	306 5	56 5	8 5	96 4	89 5	18 0
L (M) 17	5	2 0	200	36 0	240 0	51 6	12 6	53 8	73 1	75 0

(¹) Patient with periodical agranulocytosis

Increasing doses give increasing effects on immature gonads as well as on the uterus, prostate and seminal vesicles   Ovarian weight followed uterine weight with every extract of both the postmenopausal and normal male or female urine But, while the uterine weight appears first to rise to a peak and then come to a stop at some level which may or may not represent an optimal value, the ovarian weight rose progressively   These responses were similar to those due to injection of pituitary extract but not to those due to injection of pregnancy urinary extract However, it will be seen that this interpretation might be incorrect if compared with morphological results

The microscopic examination of reproductive organs and of vaginal smears of the animals injected with large doses of extract showed a usually increased growth and activity of those organs   With the equivalent of 200 c c of urine the enlargement of the ovaries was chiefly due to an increase in the number of developing follicles (Fig 1 and 2)   An ovarian response was observed within 120 hours by which time an enormous number of mature uniform follicles developed, but only to degenerate (Fig 3)   Many follicles reached almost simultaneously the Graafian preparatory stage to ovulation, but the actual rupture did not occur   All large follicles showed advanced degenerative changes involving the destruction of the ovum by pyknosis and chromatolysis   Some

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EXPLANATION OF PLATES

FIG 1 —The ovary of immature rat injected with concentrate equivalent to 200 c c of urine of a girl with periodical agranulocytosis (× 20)

FIG 2 —The ovary of immature rat injected with concentrate equivalent to 200 c c of post-menopausal urine (× 20)

FIG 3 —High power view (× 300) of section shown in Fig 1

FIG 4 —Ovarian follicle of immature rat injected with concentrate equivalent to 200 c c of postmenopausal urine, showing macrophages containing trypan blue (× 265)

FIG 5 —The ovary of immature rat injected with concentrate equivalent to 400 c c of post-menopausal urine (× 20)

FIG 6 —High power view (× 215) of section shown in Fig 5

FIG 7 —Seminiferous tubules of immature rat injected with concentrate equivalent to 200 c c of normal male urine (× 230)

FIG 8 —Seminiferous tubule of immature rat injected with concentrate equivalent to 200 c c of postmenopausal urine (× 365)

FIG 9 —Cervical epithelium of immature rat injected with concentrate equivalent to 400 c c of postmenopausal urine (× 265)
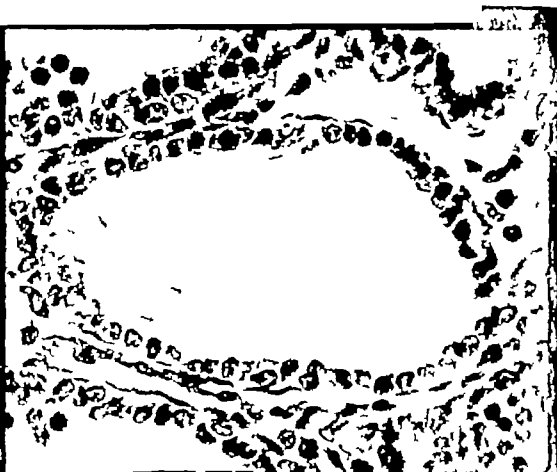
FIG 10 —Cornual epithelium of the same rat (Fig 9) (× 365)

FIG 11 —Ventral lobe of prostate of immature rat injected with concentrate equivalent to 200 c c of postmenopausal urine (× 320)

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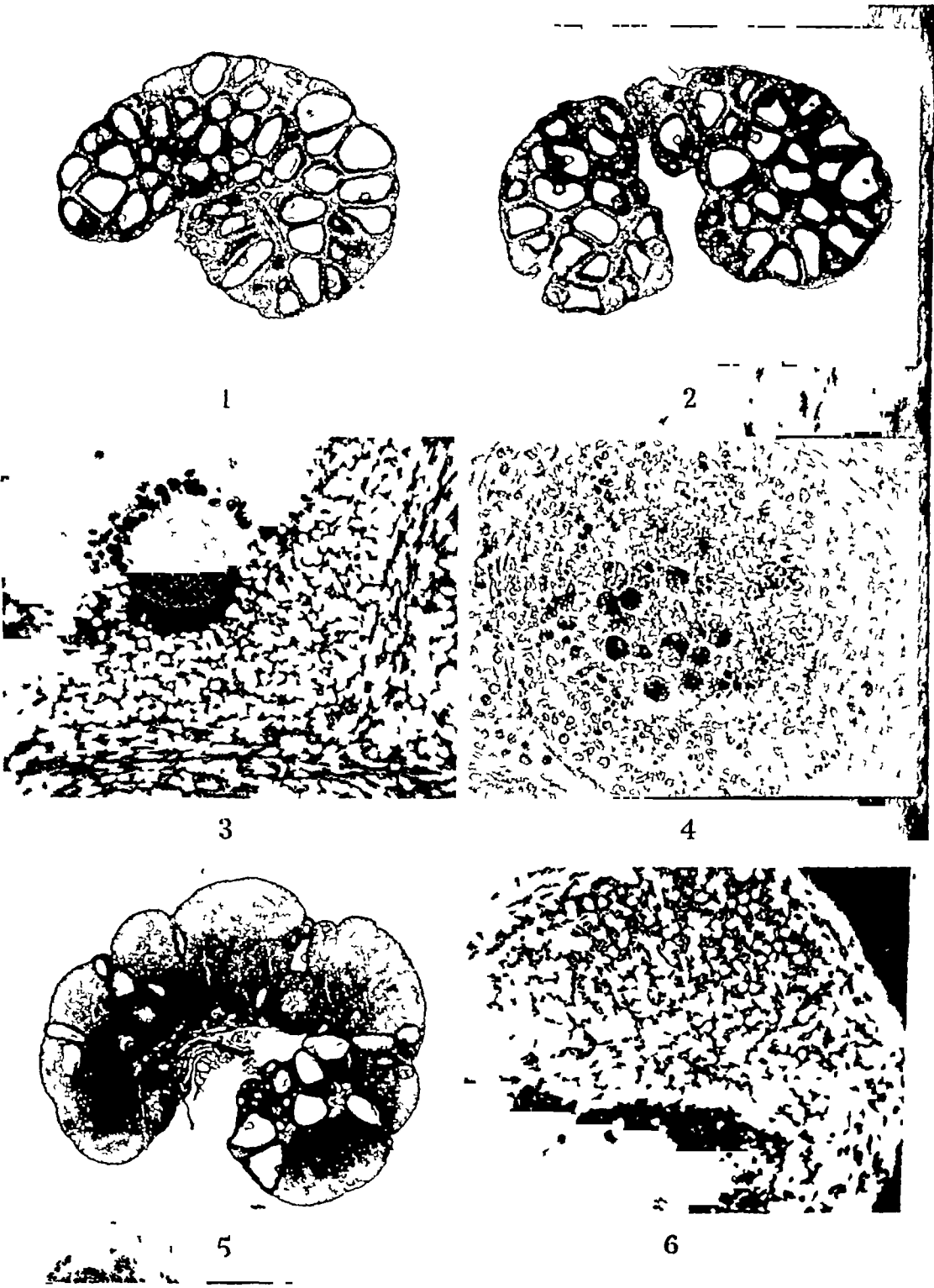
ova had become detached from the cumulus and lay free in the antrum, in some a polar spindle being present   Cytolysis of the granulosa was observed, the fragments being dispersed into the liquor of the follicles which occasionally were almost deprived of granulosa by this process   Some follicles contained macrophages packed with fragments of nuclear debris and with trypan blue if the animal was vitally stained (Fig 4)   No corpora lutea were found   Only a few primary follicles containing 2 to 7 layers of granulosa cells remained unchanged beneath the tunica albuginea   It was found difficult to differentiate the interstitial cells (gland) as such   With the equivalent of 300 to 400 c c of urine a definite thecal luteinization in various phases was observed   The theca interna thickened, the lumina of some follicles being rapidly vascularizated and obliterated (Fig 5 and 6)

The enlargement of testes was chiefly due to the development of seminiferous tubules with active spermatogenesis, but again with large doses degeneration occurs within 72 hours   Some tubules showed perfectly normal structure There was a layer of spermatogonial cells followed by a layer of spermatogonia in various stages of meiosis, followed again by spermatocytes showing first stages of spermiteleosis (Fig 7)   Spermatogenesis was never seen to end in production of fully differentiated sperms   On the other hand, some tubules showed a completely different picture   Near to the basilar membrane there were Sertoli's cells in usual number and between them spermatogonia, all having nuclei in resting state none of these being of the next stages of spermatogenesis (Fig 8) In the lumina of those tubules there was seen an eosinophylic fibrinous irregular framework which resembled the syncythial structure of Sertoli's cells   No significant changes were observed in the interstitial cells of Leydig

The uterus, prostate and seminal vesicles were much enlarged, the uterus occasionally distended with fluid achieving sometimes a 10- to 12-fold size   The most interesting feature observed was a stimulated growth of the cervical epithelium (Fig 9) and a marked enlargement of cornual glands (Fig 10)   The prostate and seminal vesicles showed an active secretory state close to sexual maturity as judged by distension of the lumina and the light areas (More, Price and Gallagher, 1930) typical of the normal secretory cells of Bouin-fixed ventral prostate (Fig 11) and the tall columnar epithelium and heightened folds of the seminal vesicles   With the technique used, the typical " secretion granules " of the active cells of seminal vesicles (Moore, Hughes and Gallagher, 1930) were almost undistinguishable

The release of oestrogen was in addition indicated by the vaginal changes and smears   The immature vagina opened regularly 4 or 5 days after the first injection of large doses with a pro-oestrus smear which changed to a cornified type

The morphological changes observed indicate that both follicle-stimulating and luteinizing effects were present in those animals and that with excess of urinary concentrates at the same time both follicle stimulation and follicular atresia in various phases were produced, and in the testes the spermatogenesis suppressed

Quantitative bioassay

The results confirm that the mouse uterus is much more sensitive to both postmenopausal and normal (male or female) urinary gonadotrophins than the

TABLE III—*Quantitative Assay of Normal Urinary Gonadotrophins in the Immature Mouse*

Subject, Sex, Age (M) = male (F) = female 24 hour output c c	No mice	Treatment		Necropsy Mean weight of			Increase over controls		Mouse uterus unit per 24 hour output M U U
		Total dose c c	Equivalent of urine c c	Body g	Ovaries mg	Uterus mg	Ovaries per cent	Uterus per cent	
	10	—	—	10 6	4 2	5 8	—	—	
C (F) 65 (950)	2	0 045	1 8	9 6	4 5	6 0	7 1	3 4	300
	5	0 060	3 2	9 5	5 0	11 6	19 0	100 0	
	5	0 075	3 9	11 1	5 2	14 0	23 8	141 3	
	4	0 090	4 7	10 0	5 4	28 7	28 5	394 8	
	3	0 120	6 3	11 0	6 0	35 0	42 8	503 4	
T (F) 70 (910)	5	0 150	7 6	9 3	5 9	30 8	40 4	431 0	
	5	0 225	11 4	9 4	9 4	54 0	123 8	831 0	
	5	0 300	15 2	9 4	9 8	58 8	133 3	913 7	
	5	0 450	22 7	9 7	10 6	61 8	152 3	965 5	
	5	0 600	30 3	9 6	12 0	62 4	185 7	975 8	
	5	0 900	45 5	10 0	13 8	63 0	228 5	986 2	
L (M) 17 (1100)	3	0 3	18 3	11 0	—	15 0	—	158 6	60
	4	0 6	36 7	11 2	—	24 5	—	324 1	
	4	0 9	55 0	11 0	—	27 2	—	368 9	
	2	1 2	73 3	11 0	—	37 0	—	537 9	
	2	1 8	110 0	11 5	—	43 5	—	650 0	
Y (F)([1]) 16 (940)	4	0 6	31 3	9 0	—	8 2	—	41 3	
	4	0 9	47 0	9 2	—	8 5	—	46 5	
	4	1 2	62 6	9 2	—	13 2	—	129 3	15
	4	1 8	94 0	10 0	—	19 2	—	231 0	

([1]) Nullipara with regular menstrual cycle   The sample was collected on the 8th day after the beginning of menses

TABLE IV—*Recovery of Postmenopausal Gonadotrophins Added to Saline and Normal Female Urine*

(Assay in immature mice)

Subject, Age 24 hour output c c	No mice	Treatment		Necropsy Mean weight of		Increase over controls Uterus per cent	Mouse uterus unit per 24 hour output M U U
		Total dose c c	Equivalent of urine c c	Body g	Uterus mg		
	4	—	—	9 1	5 8	—	
(a) C 65 (1100)	3	0 060	3 7	10 0	14 5	150 0	300
	3	0 075	4 6	11 0	17 0	193 1	
	3	0 090	5 5	8 5	21 0	262 2	
(b) Y 16([1]) (680)	3	0 6	57 9	10 0	19 5	236 2	>10
	3	0 75	67 6	10 5	33 0	468 9	
	3	0 9	86 9	10 0	51 0	779 3	
Recovery of gonadotrophins (a) from saline	3	0 075	4 6	10 3	14 3	146 5	240
	3	0 090	5 5	10 3	16 3	181 0	
	2	0 120	7 3	10 2	20 5	253 4	
	2	0 180	11 0	10 5	24 5	339 6	
	2	0 3	18 3	10 0	52 5	805 1	
Recovery of gonadotrophins (a) from urine (b)	3	0 60	3 7	12 0	13 6	134 4	300
	3	0 075	4 6	10 3	15 5	167 2	
	2	0 090	5 5	10 0	19 5	236 2	
	2	0 120	7 3	12 0	24 7	343 1	
	2	0 180	11 0	11 0	57 5	891 3	

([1]) Sample collected on the 11th day after the beginning of menses

rat's uterus and rat's ovary (Table III) On the other hand, increasing doses give increasing effects on the uterus and ovaries exactly as was observed in immature rats The increase in ovarian weight followed the increase in uterine weight When the uterine weight reached a plateau level, the ovarian weight still rose progressively It was found that to increase the weight of uterus by 100 to 150 per cent over that of the untreated controls the equivalent to 3 2 to 3 9 c c of urine was necessary for the postmenopausal urine, but about 5 times more for male urine (18 3 c c or less) and about 20 times as much for normal female urine (62 6 c c or less) These figures as well as recovery figures (Table IV) show the reliability of the results obtained with the kaolin-adsorption method It was possible to recover 80 to about 100 per cent of the original activity of postmenopausal gonadotrophins precipitate added to saline and normal urine and then putting the mixture through the kaolin-adsorption procedure

DISCUSSION

The original method of Zondek (1930) achieves only a five-fold concentration and therefore is not adequate for the detection of minute quantities of hormones present in the urine of normal men and women (Hoffmann, 1944) Leonard and Smith (1934) elaborated Zondek's method for the qualitative detection of the follicle-stimulating principle of postmenopausal urine Frank, Salmon and Friedman (1934) introduced the use of acetone instead of alcohol The dialysis of the final extract introduced by Heller and Heller (1939) and Heller and Chandler (1942) diminished considerably the toxicity of the extract for immature rats but this procedure (dialysis, reconcentration by evaporation, etc) may lead to an appreciable loss of activity (Levin, 1941) In the preliminary experiments dialysis was carried out in this laboratory but was not satisfactory Varney and Koch (1942) showed that dialysis causes more loss of activity than extraction or reprecipitation To lessen the toxicity of the extract, Frank and Berman (1939) and Frank (1939) reintroduced the acid alcohol method, using 400 c c of urine for examination They believe that the tannic acid and benzoic acid methods concentrate mainly or exclusively the follicle-stimulating substance and that the acetone and acid alcohol methods, on the other hand, concentrate both follicle-stimulating and luteinizing substances Varney and Koch (1942) elaborated a method of extraction of the crude alcohol precipitate with 50 per cent alcohol and reprecipitation from the solution with 95 per cent alcohol They claim that this modification yielded higher gonadotrophin content and less toxic extracts than others The tannic acid precipitates (Levin and Tyndale, 1936, 1937) have been found even more toxic (Heller and Heller, 1939 , Varney and Koch, 1942) The adsorption on benzoic acid causes a considerable loss of gonadotrophic activity and was later abandoned (Katzman and Doisy, 1937) In general, precipitation with alcohol has proved more satisfactory (Klinefelter, Albright and Griswold, 1943) and gives higher yields (Varney and Koch, 1942), than precipitation with tannic acid or adsorption on benzoic acid

The kaolin-adsorption method involves no special detoxicating procedure, nor any chemical fractionation of extract The crude precipitate freed from acetone and ether, if dissolved in saline was found harmless to the immature rat, even in a single subcutaneous dose of 1 c c (equivalent to 200 c c of urine)

The yields were as high or higher than those obtained with other methods   The recovery experiments proved the reliability of the results obtained with method used   It is unlikely that the extract does contain enough oestrogens or androgens to produce effects directly or indirectly

The duality of pituitary gonadotrophins as originally suggested by Fevold, Hisaw and Leonard (1931) and supported by Chow, Greep and van Dyke (1939) has been fairly well established by the isolation of pure luteinizing (interstitial cell-stimulating) protein from sheep (Li, Simpson and Evans, 1940a, b, 1941, 1942), and pig (Shedlovsky, Rother, Greep, van Dyke and Chow, 1940) pituitary, as well as by purification of the follicle-stimulating (gametogenic) hormone from pig pituitary (Greep, van Dyke and Chow, 1940)   On the other hand, there is no such evidence for the presence of both gonadotrophins in the normal male or female urinary extracts

The identification of gonadotrophins of the crude urinary concentrates in these experiments could be only approximate   The results of all tests were in general agreement and there can be little doubt that gonadotrophins were the main active factors   There is no easy way of estimating two gonadotrophins separately if they are both present in the same extract   Although the two hormones have not been separated it seems probable that postmenopausal and normal urinary concentrates contain both the follicle-stimulating and luteinizing substances   The morphological changes observed in various organs in these experiments on intact immature animals provide objective and direct evidence for both follicle and seminiferous tubules stimulation and luteinizing effects but it is obvious that those results obtained on intact immature animals are complicated by the release of both gonadotrophins by the animals' own pituitary (Noble, Rowlands, Warwick and Williams, 1939)

It was shown that the postmenopausal and normal male or female urinary extracts caused a marked growth of large uniform follicles, but none of those at the Graafian stage had ovulated and no corpora lutea could be seen after five days of injection   These results are in agreement with those of Leonard and Smith (1934)   The large doses of these extracts (equivalent to 300 to 400 c c of urine or more), on the other hand, stimulated not only a great follicular growth but also an extensive thecal luteinization accompanied with cytolysis of the granulosa and ova, the changes being various phases in advanced follicular atresia   These morphological changes are similar to those observed in intact immature animals treated with the extracts of human pregnancy urine and pregnant mare serum, except that there is no such follicular growth in animals treated with pregnancy urine   By analogy, these urinary extracts injected into the intact immature male rats may produce advanced spermatogenesis but again never to a stage of fully differentiated sperms

The animal tests may be divided according to whether the effect on the intact immature or hypophysectomized animal is primary or secondary   There are no available standards for expressing the results of the bioassay quantitatively in international units   The results are stated in various animal units such as rat units or mouse units arbitrarily defined in terms of the amount necessary to produce a defined response under defined conditions   It is convenient to speak of the bioassay of " follicle-stimulating " and " luteinizing " hormones which in varying amounts are probably present in each urinary extract, but first it should be proved that human urine contains more than one gonadotrophin

and made clear whether the follicle-stimulating hormone produces in female rats the primary gametogenic effect only as it does in male rats or both the primary and secondary (via oestrogen release) effects   The reason for choosing the mouse uterine technique (Levin and Tyndale, 1936, 1937) for the " total " gonadotrophins assay was the much greater sensitivity of the mouse uterus to the active substances and the greater accuracy and uniformity of the response to increasing doses of the extracts (Evans, Hines, Varney and Koch, 1940 , Varney and Koch, 1942)   The uterine response was produced by smaller doses of extract than those necessary to produce follicular enlargement and an increase in ovarian weight   It was found that the equivalent to 3 2 to 3 9 c c of post-menopausal urine was sufficient to increase uterine weight by 100 to 150 per cent over that of the untreated controls, while to increase the ovarian weight by 100 per cent the equivalent to 11 4 c c of urine was necessary   Both uterine and ovarian weight can be easily assayed with the same extract   Finally, reducing the assay to 72 hours after the first injection seems to be an important factor in minimizing toxic effects

SUMMARY

The kaolin-adsorption method as modified for concentrating normal urinary gonadotrophins was found an efficient, simple and quick procedure, the relatively non-toxic concentrates being suitable for further purification, isolation and standardization of the hormones   The figures for various quantitative tests seem to prove the reliability of the results obtained with the method used

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THE TISSUE RESPONSE TO HEAT-KILLED STREPTOCOCCI IN THE SKIN OF NORMAL SUBJECTS, AND IN PERSONS WITH RHEUMATIC FEVER, RHEUMATOID ARTHRITIS, SUBACUTE BACTERIAL ENDOCARDITIS AND ERYTHEMA NODOSUM

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NUMEROUS workers have in the past injected streptococcal extracts or filtrates into the skin of patients suffering from infections with haemolytic streptococci, from rheumatic fever, or from wholly unrelated conditions Swift, Wilson and Todd (1929) used crude filtrates from 10-day cultures of indifferent or σ-haemolytic streptococci, Birkhaug (1929) used culture filtrates, auto-lysates, and whole cocci from β-haemolytic, σ-haemolytic and indifferent strains of streptococci, Gibson and Thomson (1935) used an extract of ground-up haemolytic streptococci, Lyttle, Seegal and Jost (1935) used a streptococcal nucleoprotein fraction, and Taran, Jablon and Weyr (1944, 1945) used "purified M proteins" from 40 different types of Group A streptococci These workers all studied the skin reactions produced, as evidenced by appearance of odema and erythema From their results it appears that a considerable proportion of normal persons, and a higher proportion of patients with rheumatic fever or with non-rheumatic haemolytic streptococcal infections, will give skin reactions to some or all of the above streptococcal products The intensity of reaction is greater during active disease than during convalescence, and both in control and rheumatic or streptococcal groups the frequency and intensity of positive reactions increases with age Furthermore, although there are undoubted variations in the intensity of reaction produced by extracts from different strains

of streptococcus, there appears to be a wide overlap between different strains, nearly all being capable of giving reactions in some persons

In none of the above studies is there any mention of histological changes at the sites of injection It seemed to us to be of interest to study the early reactions and, more particularly, the later histological changes in human subjects injected intracutaneously with whole streptococci of different antigenic constitutions which had been killed by gentle heating Owing to the liability to cause sensitization by their very injection into the skin, it was necessary to inject the different strains which were to be tested all on the same occasion It was not practicable, without losing the co-operation of our experimental subjects, to inject more than seven stock strains These were all derived originally from human sources, and were selected so as to represent Lancefield groups A, B, D and three different Griffiths types, with and without M and T antigens A strain of *Str viridans* was also included The organisms were grown in a medium containing no proteins other than human, in order that complications due to foreign serum or Forssman type antigens should be avoided

In a few instances patients were tested with strains of non-haemolytic and σ-haemolytic streptococci derived from blood cultures of cases of subacute bacterial endocarditis These strains (Ew, Cu, Pr, Cr, Th) were cultured in small amounts under the same conditions as our standard strains In one instance a patient (Y) with rheumatic fever was tested with a strain of β-haemolytic streptococcus derived from his own throat cultures

Selection of patients

The normal subjects were members of the medical staff or students, and were carefully questioned for any history of rheumatic disease or of streptococcal infection within recent years The remainder were patients in the wards Those with rheumatic fever, rheumatoid arthritis and post-streptococcal erythema nodosum were tested during the period immediately following acute manifestation of their disease The patients with subacute bacterial endocarditis were tested before receiving penicillin treatment

EXPERIMENTAL

Selection and culture of streptococcal strains

The following strains were obtained through the kindness of Dr Dora Colebrook

No	Designation	Haemolysis on horse blood agar	Group	Type and antigen
1	B 133	Non-haemolytic	D	
3	Chown	Viridans		
6	848	α-haemolytic	B	
8	SF 130	β-haemolytic	A	I (M)
9	Smithers	Weak β-haemolytic	A	I (M and T)
11	Wheatley	β-haemolytic	A	4/24 (T)
13	R 491	,,	A	14 (T + M)

All strains were passaged three times through mice, and were then grown at 37° C for 16–18 hours in 1 litre of nutrient broth with addition of 2 per cent

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glucose, 2 per cent Na glycerophosphate, 0 5 per cent Marmite, and 10 per cent human ascitic fluid   A very heavy growth was obtained   The streptococci were centrifuged down, washed with saline, and suspended as a thick suspension in M/30 phosphate buffer, pH 7 2   The suspensions were heated at 56° C for 1 to 3 periods of 1 hour, until a heavy inoculum into broth gave no growth on incubation   They were stored as thick suspensions frozen at − 15° C   For inoculation a fresh dilution was made in sterile M/20 phosphate buffer pH 7 2 so as to contain approximately 500 × 10^6 organisms per ml   The volume injected into the skin was 0 1 ml , i e 5 × 10^7 organisms

Technique of inoculation into human subjects

0 1 ml of each streptococcal suspension, containing approximately 5 × 10^7 organisms, was injected into the skin of the inner side of the forearm in a standard pattern   The areas of erythema and oedema were measured, and a subjective estimate made of the intensity of oedema, 1, 2 and 3 days after   On the 11th to the 15th day (or occasionally later), when small firm nodules could generally still be felt at the sites of inoculation, areas of skin about 10 × 7 mm were excised under local anaesthesia and placed immediately in Zenker's solution

After fixation they were bisected, embedded in paraffin and examined in serial or step sections stained with haematoxylin and eosin , some sections were stained with Giemsa and some with Gram

RESULTS

Erythema and oedema

The erythema and oedema were maximal on the 2nd or 3rd day, and had faded away in most cases by the 4th or 5th day   When pustule formation and local necrosis occurred the contents of the pustule were invariably sterile, and the cells were predominantly neutrophil polymorphonuclear leucocytes

In order to arrive at some quantitative assessment the intensity of oedema was graded subjectively from ± to +++, and a numerical value from 1 to 6 assigned to correspond with the grades

EXPLANATION OF PLATES

Fig 1 —Skin of a normal volunteer   Intracutaneous inoculation of heat-killed streptococci, stock strain No 9   Excised after 11 days   Perivascular collection of lymphocytes and larger mononuclear cells   × 210

Fig 2 —Skin of a patient (M Wi-) with rheumatoid arthritis   Intracutaneous injection of heat killed streptococci, stock strain No 6   Excised after 9 days   Nodule showing conglutinated collagen fibres   × 210

Fig 3 —Skin response of a patient (F Ew-) with subacute bacterial endocarditis to intradermal injection of heat-killed autogenous streptococci   After 16 days   " Focal oedema " —group of swollen and vacuolar cells   × 420

Fig 4 —The same case   Thickening and conglutination of collagen fibres interrupted by swollen and vacuolar cells   Mallory preparation.   × 420

Fig 5 —The same case   " Focal oedema " with formation of nodules   × 140

Fig 6 —The same case   One of the nodules consisting of conglutinated, swollen and vacuolar cells   × 225

Fig 7 —Skin response of a patient (M B) with Rheumatic fever , response to streptococcus 11, on the 18th day   A vessel in the cutis with fibrinoid necrosis in one part of the circumference   × 400

Fig 8 —The same   An area showing fibrinoid conglomeration of the collagen   × 400

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Humphrey and Pagel

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Humphrey and Pagel
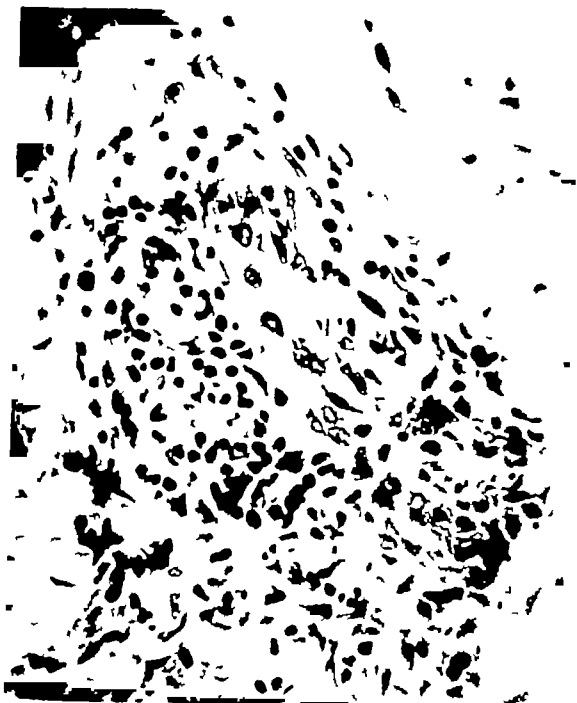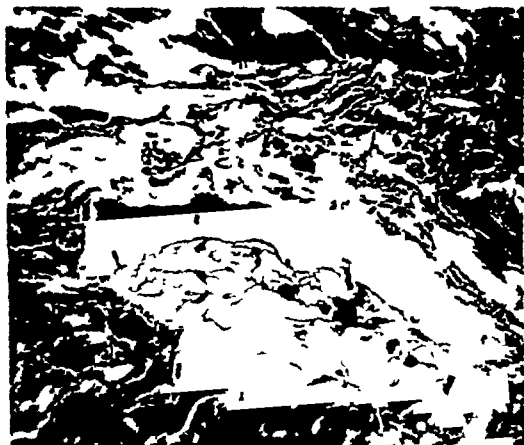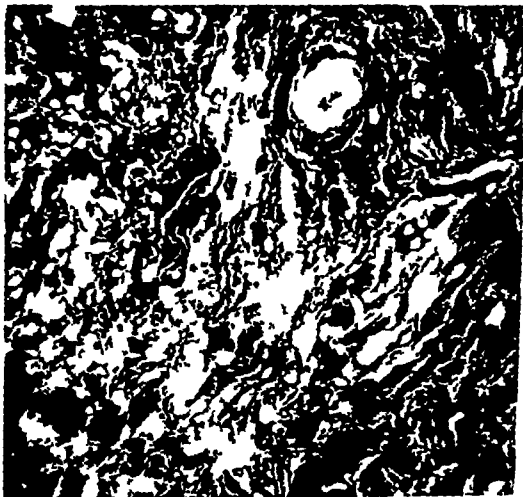
The infoimation concerning the area and intensity of the reaction to different strains, and the incidence of pustule formation, is summarized diagrammatically in Fig 9   The mean values only are represented, but there was a considerable
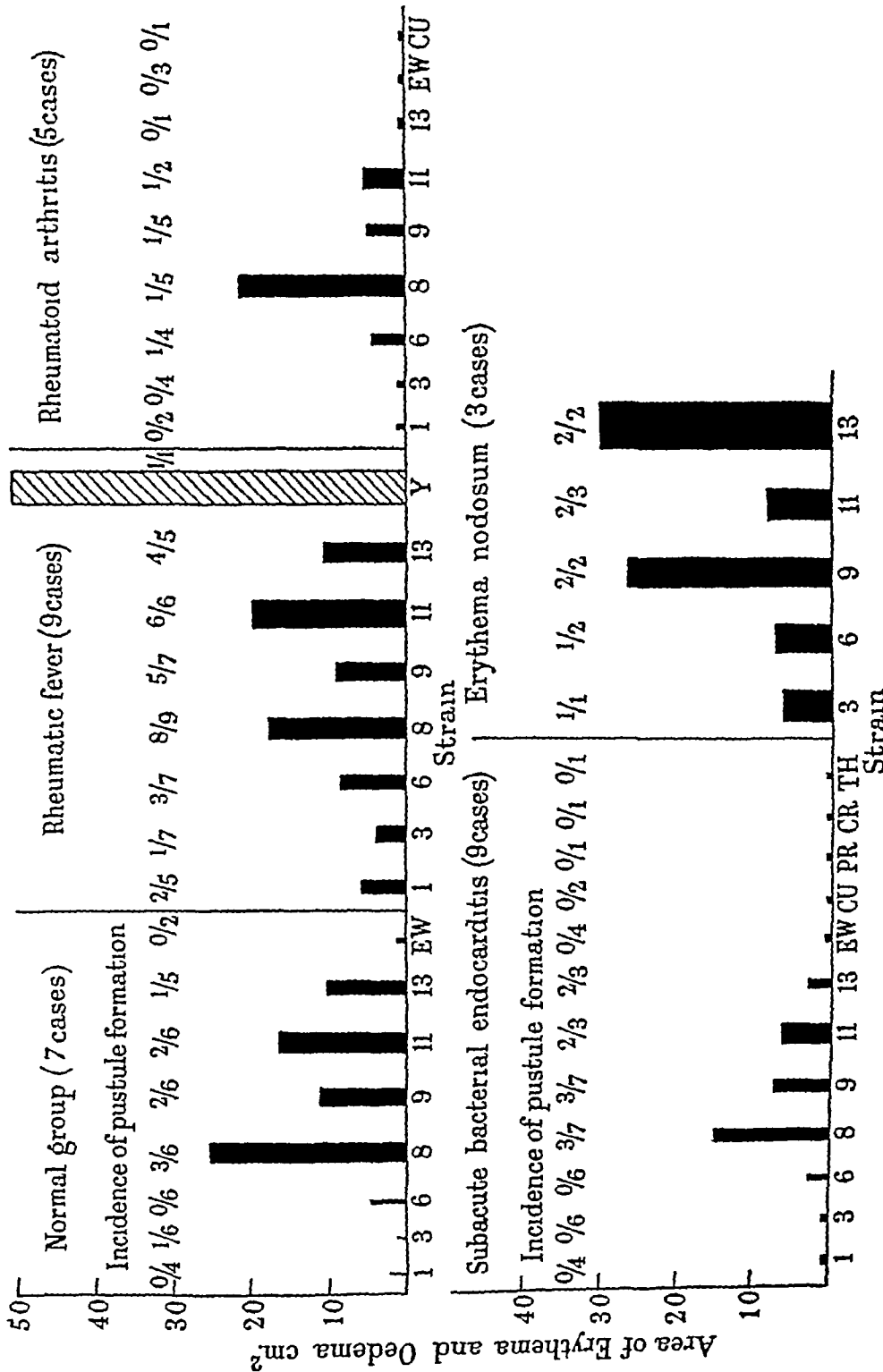

Fig 9 —Area of reaction, intensity of reaction, and incidence of pustule formation   The breadth of the columns indicates the average of intensity of oedema

scatter inside each group, and the numbers are too small for statistical analysis It will be observed that in normal subjects the Group A strains (8, 9, 11 and 13) gave rise to moderate reactions   In the rheumatic fever group the reactions to all strains were somewhat increased, and in the erythema nodosum group they were greatly increased   The patients with rheumatoid arthritis reacted rather less intensely than the normal group, and those with subacute bacterial endo carditis gave very small reactions even with their endogenous strains

Apart from the fact that the Group A strains gave the most intense reactions, there was no obvious correlation between the severity of the reactions and any of the known antigenic components of the streptococci

HISTOLOGICAL FINDINGS

Normal cases

Six normal persons were examined

The histological findings consisted of small perivascular and perifollicular collections of lymphocytes, a few eosinophils and macrophages, the latter containing cocci   There were also cocci lying free in the perivascular spaces   The latter were dilated and contained a network of fibrillary material   These " lymphangitic " changes appeared to be characteristic of the tissue response of normal individuals to heat-killed stock strains of streptococci (Fig 1 and 2)

Pathological changes

These were observed in 21 cases of 26 examined (Table I)   The central change in these cases was oedema   This appeared (a) as a diffuse infiltration of the cutis with fluid, separating the collagen fibres   The latter were often thickened and had occasionally lost their affinity for collagen stains, while the elastic and argentaffine fibres remained unaffected   (b) In some cases patchy oedema was seen with an impressive swelling and vacuolation of fibrocytes, the latter forming small " nodules " containing one or more giant cells   Such changes were sometimes accompanied by deposition of fibrinoid material in the vessel walls, along the whole circumference or confined to one side only   No cocci were seen in these areas of diffuse or patchy oedema (Fig 3 to 8)

These altered types of reaction were observed in response to one or more strains of streptococcus in all of 9 patients with rheumatic fever, in 6 of 9 patients with subacute bacterial endocarditis, in all of 3 patients with streptococcal erythema nodosum, and in 3 of 5 patients with rheumatoid arthritis (Table I) The response to other strains in these patients was of the normal " lymphangitic " types, although intermediate changes were sometimes met   There was no clear correlation between the intensity of the early erythema and oedema produced by any given strain of streptococcus and the nature of the later histological response

DISCUSSION

Although the number of cases studied was small, being limited by the difficulties attendant upon work with human volunteers, the histological findings appear to be of sufficient interest to justify their presentation   Tissue changes of the altered or pathological type are reminiscent of those seen in local serum

TABLE I —*Incidence of Abnormal Histological Changes Produced by Injection into the Skin of Different Strains of Streptococcus in Different Groups of Subjects*

Strain	Normal subjects	Rheumatic fever *	Rheumatoid arthritis	Subacute bacterial endocarditis	Erythema nodosum
1	0/4	1/5	0/2	0/2	
3	0/6	3/7	0/4	2/5	1/1
6	0/6	4/8	3/5	1/6	2/2
8	0/6	6/9	3/5	3/7	3/3
9	0/6	6/7	0/5	3/7	2/2
11	0/6	5/6	1/2	0/2	
13	0/5	2/5	0/1	0/2	
†Y		†1/1			
†Ew	0/2		1/2	†1/3	
†Cu			0/1	†1/1	
†Pr				†1/1	
†Fu				†1/1	
†Cr				†0/1	
Number of cases showing abnormal response to one or more strains	0/6	9/9	3/5	6/9	3/3

* One patient, examined at the beginning of her illness and again a year later, has been counted as two cases

† These strains were autogenous strains of α- or non haemolytic streptococci

hypersensitivity or the Arthus' phenomenon, in which extensive oedema is the most impressive structural change When an Arthus type of response is comparatively mild oedema may be " focal "—with conglutination of fibres and cells in small patches of the dermal and subcutaneous tissue without affecting the argentaffine fibres (Pagel, 1939) It may be recalled that " early rheumatic infiltration " (Klinge, 1933), i e the initial change preceding the formation of the rheumatic nodule, consists of oedema leading to separation and fibrinoid impregnation of fibrils without affecting the argentaffine reticulum fibres Undue emphasis, however, should not be laid upon a possible resemblance between the response to heat-killed streptococci and rheumatic changes

The following appear to us to be the main points which emerge from this study First, the late histological reaction affords an important supplementary indication of the type of sensitivity with which we are concerned—one, moreover, which is probably fairly independent of the initial direct " toxic " effects of the substances injected Second, the " positive " groups show a sensitized type of reaction to streptococci This was not unexpected in view of existing or preceding infection with streptococci in a number of the patients, but the variability in response to different strains is interesting Finally, the hypersensitive response in this series is of the Arthus type, although provoked by bacterial antigens It has usually been observed that such antigens elicit a delayed reaction, with predominant necrosis, in contrast to serum hypersensitivity, where there is a rapid reaction with predominant oedema (Rich, 1944)

SUMMARY

A study has been made of the histological changes observable 10–18 days or more after injection of various strains of heat-killed streptococci into the skin of normal human subjects and of patients with rheumatic fever, subacute bacterial endocarditis and streptococcal erythema nodosum

" Normal " and " hypersensitive " types of response are described, the latter having the characteristics of an Arthus-type reaction   It included focal oedema with conglutination of collagen fibres, fibrinoid infiltration of vascular walls and formation of fibrocytic nodules

" Hypersensitive " types of response were obtained with one or more strains of streptococcus in all of 9 cases of rheumatic fever, in 6 of 9 cases of subacute bacterial endocarditis, all of 3 of erythema nodosum, and in 3 of 5 of rheumatoid arthritis but in none of 6 normal subjects

Autogenous strains of streptococcus gave hypersensitive responses more frequently than the others, but otherwise there was no clear correlation between the possession of particular antigenic constitution and the type of histological response

The late histological changes were not closely correlated with the short-term reactions of oedema, erythema and pustule formation

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QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS
THE PRIMARY RESPONSE

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FOR many years both qualitative and quantitative data have been published concerning the response of different animals to diphtheria toxoid, administered in different ways, in different amounts, and with different time intervals between inoculations Since formol-toxoid and the more active adsorbed toxoids, such as alum precipitated toxoid, were themselves variable in potency (Bousfield, 1947) and prepared in different ways (Lewis, 1941), the significance of these published results could not easily be assessed

With the introduction of a purified toxoid adsorbed on to a suspension of aluminium phosphate—P T A P (Holt, 1947)— a reagent was made available in which variations in the proportion and concentration of the two components—toxoid and carrier—could be altered at will, and reliance placed on the reproducibility in the composition and antigenicity of different preparations

The characteristic response of an animal, say a guinea-pig, to a primary inoculation is well illustrated by experiments reported by Glenny and Sudmerson (1921)   These workers inoculated a number of guinea-pigs with a single dose of underneutralized toxin-antitoxin mixture   There occurred a latent period of about three weeks, followed by the appearance of circulating antitoxin, which reached a peak value in about eight weeks, followed by a fall in titre   In one such experiment the serum antitoxin titres of guinea-pigs were followed over a period of two years, and for the greater part of the latter period of observation the animals showed a constant titre of circulating antitoxin—the final baseline level of immunity   Recently Glenny and Barr (1947) have reported a similar phenomenon in horses, in which tetanus antitoxin titres were observed over a period of five years following a single inoculation of tetanus toxoid

Since it has been shown by Schoenheimer, Ratner, Rittenburg and Heidelberger (1942) that the " half-life " of a globulin molecule is two weeks, the prolonged maintenance of circulating antitoxin clearly indicates a continuous production of antitoxin by the globulin-producing cells   This conclusion is in keeping with that of Burnet, Freeman, Jackson and Lush (1941)   " it is equally conceivable that once the antigenic stimulus has been applied, the cells continue to produce antibody long after the antigen has been completely disintegrated " Glenny and Barr (1949) have expressed very similar conclusions   It also shows that the capacity to produce the altered globulin, antitoxin, as a result of an inoculation of toxin or toxoid presumably depends on a permanent alteration, or kainogenetic effect, in the pattern of the globulin elaborated by these cells Since, also, the circulating antitoxin persists for at least two years in guinea-pigs and five in horses, the original stimulus must have induced so profound a modifica-

tion in the globulin-producing cells that its effect was handed down to daughter cells, for it is unlikely that the original antibody-producing cells would survive for so long   In other words, it is suggested that the change brought about by the action of toxin or toxoid on the globulin-producing cells is one that affects the very genetics of the cells

It follows from the above considerations that a primary inoculation of toxoid stimulates a number of globulin-producing cells, which subsequently elaborate antitoxin instead of normal globulin, and continue to do so at an even tempo for a substantial period of time   The shape, therefore, of a pure primary response curve would be as drawn in Fig 1
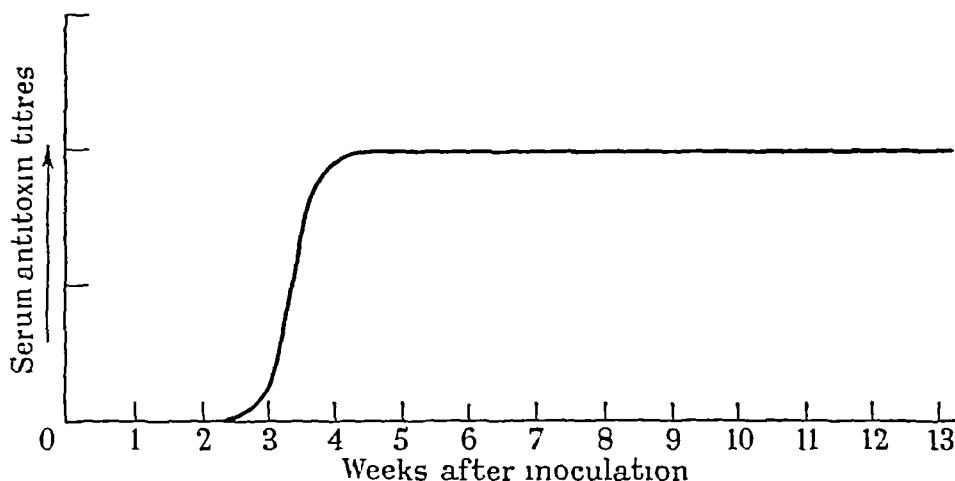
FIG 1 —Theoretical pure primary response to diptheria toxoid

Can such an ideal primary response curve be obtained ?

When formol-toxoid is employed as a primary stimulus, and the response closely followed, we may obtain one of two differently shaped curves, shown in Fig 2   The time for maximum response in the curve showing a marked decline (B) is usually 6–7 weeks, whereas for the one rising to an almost constant level (A) it is 4 weeks   The latter closely approximates the theoretical curve   It is suggested that Curve B is another example of the marked effect of the presence of traces of toxin in toxoid on the short-range antitoxin response when used as a primary stimulus (Glenny, Pope, Waddington and Wallace, 1925)   This, perhaps, owing to local fixation of the toxin in the non-immune animal, with subsequent release, would act as a *second stimulus*

Formol-toxoid has been largely displaced by alum-precipitated toxoid (A P T), which was shown by Glenny, Buttle and Stevens (1931) to be some one hundred times as potent   They ascribed this increased antigenic efficiency to a resulting slow adsorption and elimination of the precipitated toxoid

Recently the antitoxin-time-response curves have been observed in groups of not less than 12 guinea-pigs, which had received subcutaneous inoculations of 1 Lf amounts of purified toxoid adsorbed on to different quantities of pure aluminium phosphate carrier (Fig 3)

In all cases the time required to reach maximum mean antitoxin titre was the same (about 25 days), but the final base-line mean titres were different, being

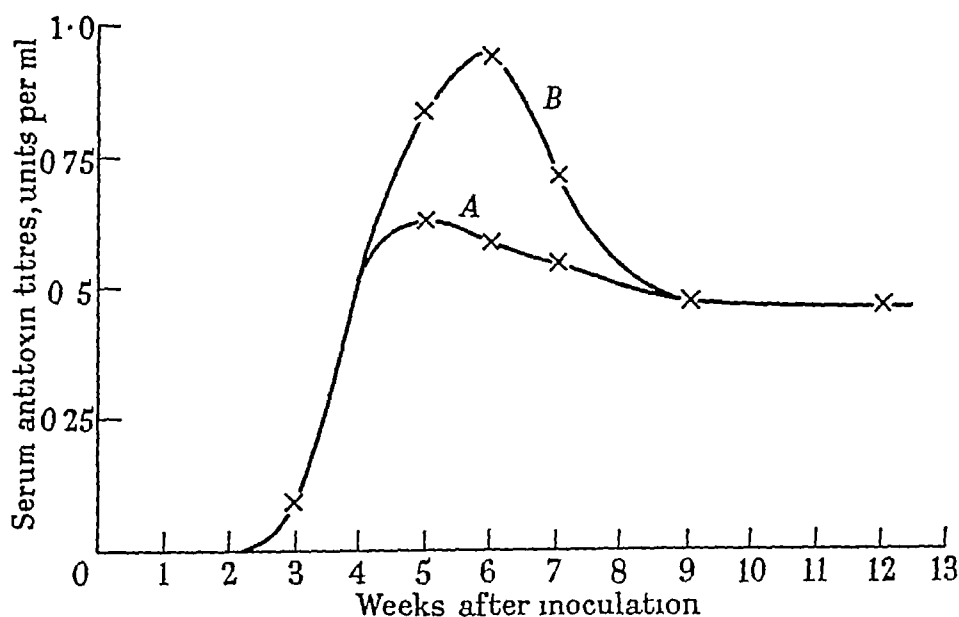higher as the amount of AlPO₄ carrier injected was increased up to the optimal amount of 3 mg (Holt, 1947)

FIG 2 —Types of primary response to diptheria formol-toxoid, e g 100 Lf
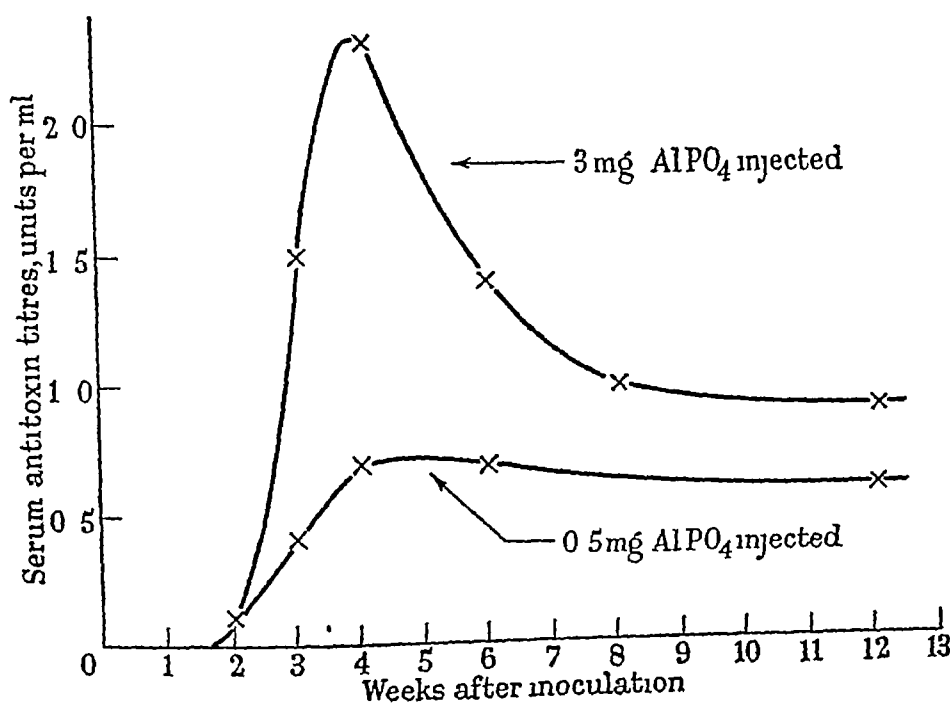
FIG 3 —Primary response to subcutaneous inoculation of P T A P (1 Lf, dose vol 0 5 ml)

When the nodules which develop after the injection of 3 mg of AlPO₄ carrier (with 1 Lf of toxoid) were surgically excised at different times after inoculation, and the antitoxin response curves again followed, it was found that there was no

significant difference in the curves when the interval between injection and excision exceeded 14 days   But when the nodules were excised 7 to 10 days after inoculation a peak titre did not occur , instead a response was obtained almost identical with the theoretical pure primary (Fig 4)   This same type of response was also observed when the carrier disappeared completely from the site of inoculation by natural means within 7 days, as is shown in Fig 3, where the response from an injection of 0 5 mg of carrier was followed

It is important to note that the levels of circulating antitoxin at 12 weeks (Fig 4) were the same whether the nodules (3 mg AlPO₄ injected) were excised
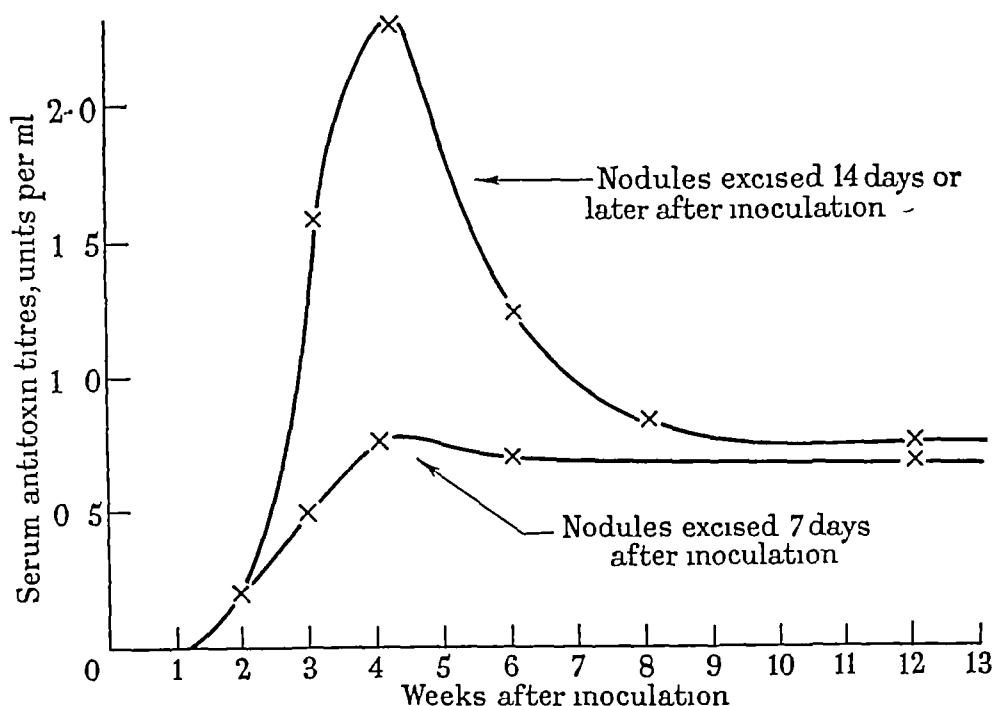
FIG 4 —Effect of excision of subcutaneous nodule on the antitoxin response   The sub cutaneous nodules were excised 1, 2, 3, 4, and 6 weeks after inoculation, controls no excisions   The responses were virtually identical in all cases except that where the nodules were excised after 7 days

after 7 days, 6 weeks, or left *in situ*   When, however, the nodules were excised before the 7th day after injection, the response was less the earlier they were removed, since the shorter the time interval, the less material is transported to the antibody-producing cells (*vide infra*)

The difference, therefore, between the responses obtained when the nodules were excised between 7 and 10 days after inoculation and those left *in situ* represents a second response superimposed on the pure primary   The immunological significance of the nodule remaining *in situ* much longer than one week is of small practical importance, since the final base-line level of immunity, i e circulating antitoxin, is unaltered by its presence   The significant time interval is the first 7 to 10 days

In addition, these findings show that a second response may be obtained as early as 10 to 14 days after a primary inoculation

An examination of the *cellular* response in guinea-pigs to moderate amounts of A P T or P T A P injected subcutaneously showed that in some 6 hours leucocytic infiltration had commenced, and was well marked in 24 hours Also, particles of carrier could be demonstrated in the granulocytes This was done by "staining" sections of the subcutaneous nodules with alumon (Aurine-tricarboxylic acid) Subsequently the mass enlarges by leucocytic infiltration, becomes rounded-off and enclosed in a fibrous capsule, and the contained granulocytes then show karyorrhexis The process of encapsulation requires 7 to 10 days (Fig 7) Then the mass contracts and the capsule thickens by increased fibrosis

When the nodules were excised *intact* 7 to 10 days after inoculation, and implanted subcutaneously into sensitized guinea-pigs, there followed only a
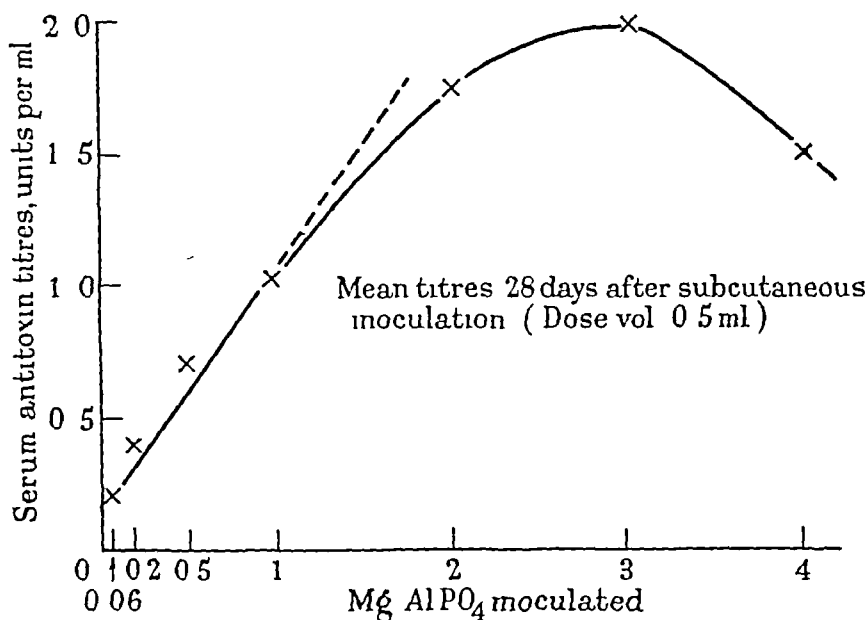
Fig 5 —Influence of amount of AlPO₄ inoculated on the antitoxin response (Dose toxoid 1 Lf)

very small second response, which may well have resulted from a slight rupturing of the delicate young capsule during the necessary surgical manipulations Such 10-day-old nodules, after being macerated, gave rise to a marked second response when inoculated into sensitized guinea-pigs

Since it has now been shown that when the residual mass of adsorbed-toxoid is excised between 7 and 10 days after subcutaneous inoculation the antitoxin response is almost identical with the theoretical primary one, and produces the same final base-line level of circulating antitoxin as a non-excised inoculum, and that fibrous encapsulation of the residual mass is complete in about the same time, it is concluded that neither the second response phenomenon, nor delayed adsorption of the antigen (depot hypothesis of Glenny, Buttle and Stevens, 1931), is responsible for the enhanced antigenicity of adsorbed toxoid when used as a primary stimulus

A comparison has been made of the antigenic efficiency of samples of P T A P containing different amounts of AlPO₄ carrier and employing both the sub-

cutaneous and intramuscular routes   When 2 5 mg AlPO₄ in P T A P was inoculated subcutaneously in young children, the incidence of palpable nodules was 90 per cent, and 100 per cent when larger amounts were inoculated (Bousfield, 1947)   The same amounts of AlPO₄ injected intramuscularly invariably produced the better Schick conversion rates (Holt and Bousfield, 1949)   It is suggested, therefore, that the reason for the increased Schick conversion rate among those children which received the P T A P inoculations intramuscularly is that the continuous movement of the muscle delayed or even prevented fibrous encapsulation of much of the material injected, although this is not readily demonstrable   This would result in a greater fraction of the injected mass reaching the antitoxin-producing cells
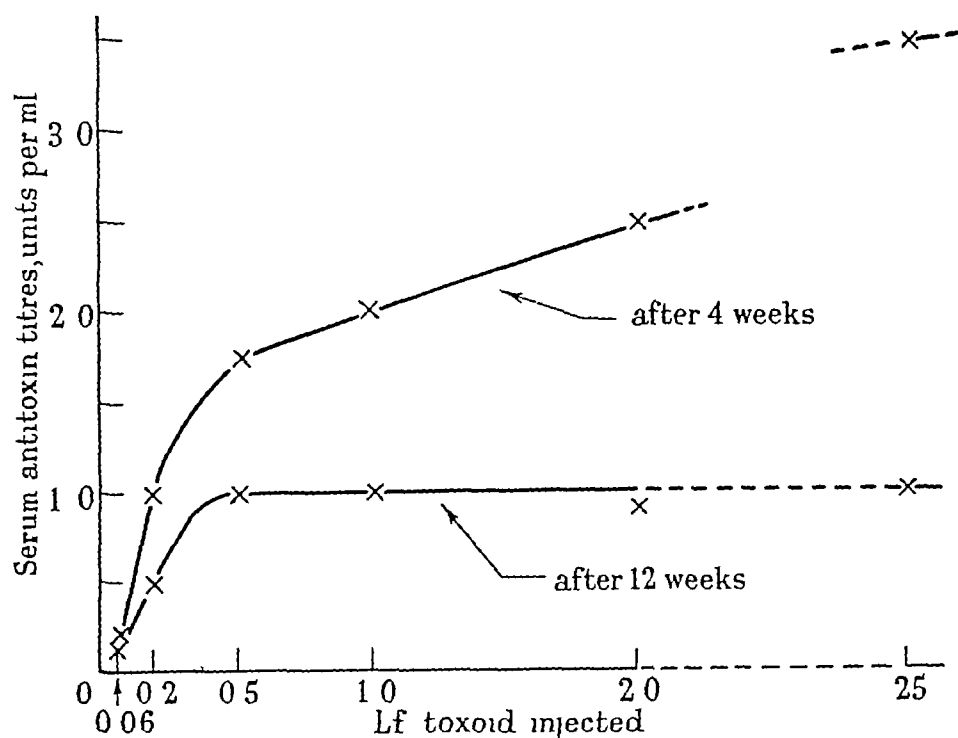
FIG 6 —Antitoxin response, in guinea pigs, AlPO₄, carrier constant at 3 mg   Injected Dose of toxoid varied

It is suggested that the reason for the enhanced antigenicity of A P T and P T A P is that the mineral carrier conditions the amount of toxoid that reaches the antitoxin-producing cells via the granulocytes

An examination of the cellular response in guinea-pigs to subcutaneous injections of aluminium phosphate greater than 2 5 mg revealed that, instead of complete and thorough infiltration of the mass by leucocytes as described earlier for smaller amounts, the granulocytes had succeeded only in penetrating the periphery of the mass

When the injection volume is 0 5 ml and the injections are made subcutaneously on the ventral wall, the guinea-pig can remove completely, without a residual nodule, slightly less than 1 mg of aluminium phosphate   Quantities greater than 1 mg invariably give rise to the development of well-defined subcutaneous nodules

FIG 7.—Section of guinea-pig skin, 10 days after subcutaneous inoculation of 0 5 c c A P T   (Approximately 2 mg of mineral injected)

Note Fibrous encapsulation of residue of injected material—the formation or the 'nodule'

The antitoxin responses in guinea-pigs, 28 days after the subcutaneous inoculation of a constant amount of purified toxoid (1 0 Lf) adsorbed on to increasing amounts of AlPO₄ carrier, showed that, with amounts up to 1 mg, the response was directly proportional to the amount of carrier used When between 1 and 3 mg were injected the slope was less steep, and progressively larger nodules formed The injection of more than 3 mg of AlPO₄ carrier (in 0 5 ml) resulted in a submaximal antitoxin response (Holt, 1947) Although much of the higher values was due to a second response effect (Fig 3 and 4), the 3 mg inoculation gave the highest base-line level of circulating antitoxin It is suggested that, in fact, more carrier is phagocytosed when 3 mg is injected subcutaneously than when greater or less amounts are used, 3 mg being the point of maximum advantage in respect of two opposing forces—amount of carrier to be phagocytosed and loss of carrier by encapsulation

If this interpretation is correct, viz the antitoxin response to samples of P T A P is conditioned by the number of particles of carrier which, with their charge of adsorbed toxoid, are phagocytosed and escape to reach the globulin-producing cells, the limit being the receptive capacity of the animal employed, then the maximum response to an inoculation of 1 Lf of toxoid would only be obtained when the toxoid is distributed among the globulin-producing cells in such a way that each cell that receives toxoid receives one minimal stimulating dose

An experiment was carried out employing different amounts of purified toxoid, adsorbed on to 3 mg of AlPO₄, the amount of carrier which had been found to be optimal for 1 Lf of toxoid Within the limits of 0 5 Lf and 25 Lf there was little difference in the antitoxin responses in guinea-pigs 4 weeks after injection, and *none* after 3 months The antitoxin response declined rapidly as amounts less than 0 5 Lf were used (Fig 6)

It is suggested that when 0·5 Lf of toxoid is adsorbed on to 3 mg of AlPO₄ the " statistical " particle, viz that quantity of AlPO₄ that reaches one globulin-producing cell, carries just one minimal stimulating dose of toxoid When less toxoid is employed, the antitoxin-producing cells fail to receive one minimal stimulating dose When, however, quantities of toxoid greater than 0 5 Lf/3 mg AlPO₄ are used, each antitoxin-producing cell would receive an excess of toxoid If one assumes that the " All or None Law "—as applies to the heart and nerves—also holds for the reactivity of antitoxin-producing cells, then those cells which receive an excess of antigen will make no greater response than those which receive one minimal stimulating dose On the other hand, summation of subminimal doses may result in a response if the time interval between the several stimuli is not too prolonged

The ratio 0 5 Lf/3 mg AlPO₄, or 0 16 Lf/mg AlPO₄, is therefore the critical ratio of toxoid to AlPO₄ carrier, for maximal antigenic efficiency in guinea-pigs

The results of a clinical investigation on similar lines has recently been reported (Holt and Bousfield, 1949)

In this inquiry Schick-positive young children were inoculated intramuscularly with samples of P T A P , the dose of AlPO₄ carrier was constant at 7 5 mg , but the amount of purified toxoid used varied The Schick conversion rates, determined 6 months later, showed no statistically significant difference in the results, whether 5 Lf or 30 Lf of toxoid were injected with the 7 5 mg of AlPO₄—limits 95 2 to 98 4 per cent Schick conversion, 634 cases examined

The lowest toxoid to carrier ratio employed was 0 66 Lf/mg AlPO₄ Efforts to determine the " human " minimal effective ratio of toxoid to carrier are now in progress, and it will be interesting to learn how closely it agrees with the guinea-pig ratio

Since 25 Lf of formol-toxoid will give rise to about 0 1 u/ml serum antitoxin in guinea-pigs, and this represents a moderate response to the antigen in simple solution, and 0 5 Lf adsorbed on to 3 mg AlPO₄ induces 1 0 u/ml which represents the response using the optimal amount of carrier, in so far as these figures are comparable it may be calculated that the antigenicity of the toxoid has been increased 500 times by adsorption in the proportion given

$$\text{Ratio} = \frac{25 \times 1\,0}{0\,1 \times 0\,5}.$$

SUMMARY.

The shape of a pure primary response curve to an inoculation of toxoid is deduced

This pure primary response may be obtained with adsorbed toxoid, by suitably timed surgical excision of the material inoculated

It is shown that the enhanced potency of adsorbed toxoid is not due to the second response phenomenon, nor to delayed release from a depot, but to a more efficient distribution of the toxoid among the antitoxin-producing cells This, it is suggested, is brought about by the *transportation* of the (adsorbed) antigen to the antitoxin-producing cells by the polymorphonuclear leucocytes which have been shown actively to phagocytose the AlPO₄ carrier used

The critical minimal ratio of toxoid to AlPO₄ carrier for the immunization of guinea-pigs has been determinated and found to be approximately 0 16 Lf/mg AlPO₄ By comparing the antitoxin response in guinea-pigs to 25 Lf of formol-toxoid with that obtained with 0 5 Lf adsorbed on to the optimal amount of carrier (3 mg AlPO₄), the maximum ratio of antigenic efficiency of adsorbed to its parent formol-toxoid, when used as primary stimuli, has been calculated to be of the order of 500

The second response phenomenon may be elicited as early as 10–14 days after primary inoculation

It was suggested by Burnet *et al* (1941) that once the antigenic stimulus has been applied, the antibody-producing cells continue to produce antibody long after the antigen has been disintegrated

It is now suggested, in respect of diphtheria antitoxin production, that this kainogenetic effect is inherited by the *daughter cells* of those originally stimulated

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STUDIES ON STAPHYLOCOCCUS MUTATION   AN INVESTIGATION OF THE GROWTH REQUIREMENTS OF A " G " (GONIDIAL) VARIANT,

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IN a previous communication (Hale, 1947) I suggested that the small colony size of the so-called " G " gonidial variants of the staphylococcus results from its requiring growth factors in excess of the needs of the parent strain   This present paper reports the results of an investigation of the growth requirements of one of these variants, selected by the use of gentian violet

METHODS

The strains used in this investigation were
    Parent Strain Au 18L—isolated from an abscess
    Variant Au 18S—isolated from Au 18L by the aid of gentian violet (Hale, 1947)
Tubes for testing growth requirements received 4 5 ml of the basic media +
0 5 ml of a dilution of the substance under test, or 0 5 ml saline in the case of the control tubes
For the preparation of an inoculum the organisms were grown for 24 hours at 37° C on nutrient agar slopes   Suspensions were made in saline, enumerated turbidometrically and dilutions made so that 1 ml   contained 5×10^3 'cocci per ml   Each tube then received 0 2 ml of this diluted suspension to give an inoculum of 1000 cocci or 200 cocci per ml media

EXPERIMENTAL

Preliminary investigation of the growth of the gonidial variant.

The commencement of growth of the variant Au 18S was delayed in nutrient broth and the final population attained was always much smaller than that of

the parent strain Au 18L   Nevertheless, it was decided for this investigation to use a simple medium that, while allowing normal growth of the parent strain, would not support, or at best give only minimal, growth of the variant   Such a medium could then be supplemented so that it gave a growth of the variant comparable to that of the parent   Analysis of these supplements should then reveal the additional factors required for maximal growth by the variant

Casein hydrolysate medium prepared according to McIlwain and Hughes (1944), providing it was supplemented by glucose, proved to be adequate for this purpose

TABLE I —*Growth of Staphylococci in Casein Hydrolysate Basal Medium*

		Incubation in hours				
		17	24	41	48	66
Casein Hydrolysate	Au 18L	—	±	+	++	++
	Au 18S	—	—	—	—	?
Casein Hydrolysate + Glucose M/50	Au 18L	±	+++	++++	+++	++++
	Au 18S	—	—	tr	tr	±

NOTE —In this and all subsequent tables   + signs indicate degrees of growth   tr = trace of growth

Sources of growth factors necessary for variant strain

Yeast extract was prepared by crumbling 1 lb of bakers' yeast into 1 litre of water at 85° C and the whole was held at that temperature for 15 min   After paper filtration to remove yeast cells the filtrate was sterilized by Seitz filtration and tested by addition to the basic casein medium

The results show that this material added to the basic medium supported a growth of the variant equivalent to that of the parent after 66 hours' incubation at 37° C   This activity was exhibited at 1/10 final dilution of the extract and some growth stimulation was noticed in higher dilutions   Precipitation of the yeast extract by lead acetate left the active material in the filtrate

Fresh human serum and human urine freshly voided and sterilized by Seitz filtration were also found to exhibit similar activity to yeast extract in promoting maximal growth of the variant in both cases at final dilution of 1/10

TABLE II —*The Effect of Yeast Extract, Serum and Urine on Growth*

	Strain	Final dilutions of substance tested				Incubation time
		10^{-1}	10^{-2}	10^{-3}	Control	
Yeast Extract	Au 18L	++++	++++	++++	++++	66 hr
	Au 18S	++++	++	+	±	
Serum	Au 18L	++++	++++	++++	++++	58 „
	Au 18S	+++	++	tr	±	
Urine	Au 18L	++++	++++	++++	++++	50 „
	Au 18S	++++	++	±	tr	

Medium = Casein Hydrolysate + M/50 Glucose

Marmite, obtained from the manufacturers free of salt and made up to a 30 per cent w/v solution, proved to be highly active   It gave growth of the variant comparable with that of the parent up to a final dilution of 1/500 and demonstrated considerable growth stimulation up to a dilution of 1/62,500

Further investigations demonstrated that the active material was extractable by acids, but not by ether, chloroform or amyl alcohol

TABLE III —*The Effect of Marmite (30 per cent w/v Solution) on Growth*

	Marmite dilutions in basal medium	Incubation in hours	
		24	48
Au 18L	10⁻¹	+ + + +	+ + + +
	10⁻²	+ + + +	+ + + +
	1/500	+ + + +	+ + + +
	1/2,500	+ + + +	+ + + +
	1/12,500	+ + + +	+ + + +
	1/62,500	+ + + +	+ + + +
	C	+ + +	+ + + +
Au 18S	10⁻¹	+ + +	+ + + +
	10⁻³	+ +	+ + + +
	1/500	±	+ + + +
	1/2,500	±	+ + +
	1/12,500	tr	+ + +
	1/62,500	tr	+ +
	C	tr	+

Using casein hydrolysate medium it was found that the control tubes containing no supplementary factors did show minimal growth after prolonged incubation, and at this stage it was thought better to substitute for this medium the synthetic amino acid medium described by Fildes, Richardson, Knight and Gladstone (1936) and modified by Fildes and Richardson (1937) and Gladstone (1937) With this medium the above results were repeated, and providing the inoculum did not exceed 200 cocci per ml of medium the control tubes showed no visible growth after 7 days' incubation, although from these control tubes the presence of viable organisms could usually be demonstrated

The effect of yeast nucleic acid on the growth of the variant

The distribution and certain properties of the active material suggested the testing of yeast nucleic acid Yeast nucleic acid was prepared according to the method described by Cole (1926) and a 5 per cent w/v solution was active in stimulating growth up to a dilution of 1/100

The result suggested the use of yeast nucleic acid hydrolysed under different conditions to give in the hydrolysed mixtures predominantly nucleotides, nucleosides or the free bases Hydrolysis was carried out under the conditions described by Levene and Bass (1931) The hydrolysed mixture that contained the free pyrimidine and purine bases was a hundred times more active than either the original yeast nucleic acid or the mixtures that contained nucleotides or nucleosides This difference was so marked that it appeared most probable that the slight growth-promoting activity of the yeast nucleic acid and the other mixtures was, in fact, due to the presence of free bases in these materials

Uracil as a " growth stimulator " of the variant

A test carried out using the individual bases known to be present in yeast nucleic acid demonstrated quite clearly that, while uracil was active in promoting growth up to a concentration of 10^{-6} M, guanine, cysteine and adenine were ineffective

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TABLE IV —*The Effect of Hydrolysis on the Growth-stimulating Properties of Yeast Nucleic Acid*

	Final dilutions of substance tested				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Control
Yeast nucleic acid .	++++	++++	++	—	—
Yeast nucleic acid hydrolysed with weak ammonia at 115° C (nucleotides)	++++	++++	+++	—	—
Yeast nucleic acid hydrolysed with 20 per cent ammonia at 150° C for 3½ hours (nucleosides)	±	++++	++	±	—
Yeast nucleic acid hydrolysed with 25 per cent H_2SO_4 2 hours (free bases)	?	++++	++++	++++	—

Test organism is the Variant Strain Au 18S
Synthetic medium 7 days' incubation
The yeast nucleic acid solution was 5 per cent w/v and the hydrolysed mixture arranged so that 5 g of yeast nucleic acid was hydrolysed and then made up to 100 ml

TABLE V —*The Effect of Uracil on the Growth of the Variant*

	Final dilutions of substance tested					
	10^{-4} M	10^{-5} M.	10^{-6} M	10^{-7} M	10^{-8} M	Control
Uracil	++++	++++	++++	+	±	±
Adenine	+	±	±			±
Cytosine	±	±	±			±
Guanine	±	±	±			±

Synthetic Medium, 7 days' incubation

DISCUSSION

The results reported above prove that the growth of the small variant, selected by the aid of gentian violet from cultures of the parent staphylococcus Au 18L, is stimulated by the addition of uracil to the chemically defined medium described by Fildes and Richardson (1937) and Gladstone (1937)   Uracil is necessary for the growth of staphylococci under anaerobic conditions (Richardson, 1936) but aerobically they are apparently able to synthesize sufficient uracil for their requirements   The variant strain reported here differs in that the aerobic synthesis of uracil is also very much impaired   This aerobic synthesis is not completely suppressed as an inoculum of the variant in the synthetic medium does grow eventually (after 7 days)   Growth of the parent strain and the variant under anaerobic conditions, when both will be dependent upon the uracil present in the medium, should therefore approximate   This is found to be the case, for nutrient agar plates of the parent and variant incubated under anaerobic conditions exhibit colonies approximately equal in size

The impaired synthesis of uracil by the variant must result in deficiency of ribo-nucleic acid   Henry and Stacey (1943) have shown that the staining properties of Gram-positive organisms depend upon the presence of magnesium ribo-nucleate ,   therefore it is not unreasonable to assume that the staining properties of the variant might differ from those of the parent   It was found that when the organisms were stained with a mixture of an acid and basic dye

the parent failed to take the acid dye until a pH of 2 0 was attained, whereas the variant changed over at pH 3 0–3 5 This is more characteristic of Gram-negative organisms where changeover takes place at pH 4 0–4 5 Gram-negative organisms are more resistant to gentian violet than gram-positive organisms (Stearn, 1927), probably because they have not the same affinity for this basic compound By the same reasoning one could account for the variant's greater resistance to gentian violet and its consequent selection in cultures containing concentrations of gentian violet just inhibitory to the parent strain

It is not claimed that uracil is the only requirement by which the variant differs from the parent Thus, addition of uracil to solid media does definitely stimulate the growth of the variant, but the colonies are still not as large as those of the parent on the same media less uracil Other substances were found which stimulated the growth of the small variant, but only if uracil was present It appears, therefore, that uracil is the most important requirement

SUMMARY.

(1) A small colony variant of staphylococcus was isolated by cultivation on media containing gentian violet

(2) The growth characteristics of the variant were shown to be dependent upon an impairment of uracil synthesis Addition of uracil to the medium stimulated growth

(3) The selective action of gentian violet in relation to uracil metabolism is discussed

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A RAPID CONCENTRATING STILL

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IN the apparatus described here aqueous solutions can be concentrated at rates of 12–14 l /hr at temperatures not exceeding 36° C Under these conditions many enzymes, bacterial toxins and other substances can be recovered in high yield No novelty is claimed for this apparatus , it is a laboratory application of the climbing film still used in industry, and it is closely modelled on an apparatus described by Mitchell, Shildneck and Dustin (1944) See also Kemmerer (1945), Ames (1946), Muirhead (1946) and Reavell (1946a, b) The apparatus is described here because it can be made from parts (mainly standard) supplied by a British firm, and because it is thought that a detailed description of its operation would be useful to those concerned with the isolation of small amounts of biologically active substances from large volumes of solution

Construction

The glass part of the apparatus (Fig 1) is constructed of " Pyrex " industrial glassware supplied by Messrs James A Jobling & Co , Ltd , of Sunderland (their Drawing No 7338/2) A is a standard 1 in Y-piece (All dimensions are internal) B is a standard Davies type double wall condenser, 35 in long, with pressed ends of 1 in bore , it is used as heat exchanger, and steam is passed through the inner and outer water jackets The heating surface has an area of 235 sq in C is a standard 1 in 90° elbow with a $\frac{5}{16}$ in side tube for holding a thermometer D was especially made from a 5 l flask with a 1½ in top neck, a 1 in bottom neck, and a 1 in neck entering horizontally at a tangent with its centre 1 in. above the middle of the flask E is a 20 in length of 1 in pipe line F is a standard 1 in T-joint G is a standard 1 in 45° elbow with a $\frac{5}{16}$ in side tube , this side tube is generally sealed with a rubber bung H is a 7⅞ in length of 1 in pipe line I is a standard 1½ in 90° elbow J was especially made from a 1 l flask with a 2 in top neck, a 1 in bottom neck, and a 1½ in neck entering the flask horizontally at a tangent in the middle K was especially made from ⅝ in tube with pressed ends of 1 in bore , for ease in assembly the vertical part of it is cut into three equal lengths, which are then connected with rubber tubing (not shown on the diagram) L is a standard 2 in U-bend M is a standard 3 in to 2 in reducer N is a standard 42 in length of 3 in pipe line All the parts listed above are held together in the usual way by bolts passing through metal joint flanges fitted with graphited asbestos inserts , rubber interface joint gaskets are placed between each pair of pressed ends

A copper condenser, obtainable from T J Maskell, 30A, Jericho Street, Oxford, drawn in Fig 2 on four times the scale of Fig 1, is inserted in tube N It consists of four 42 in copper tubes of 2½ in , 2 in , 1½ in and 1 in diameter
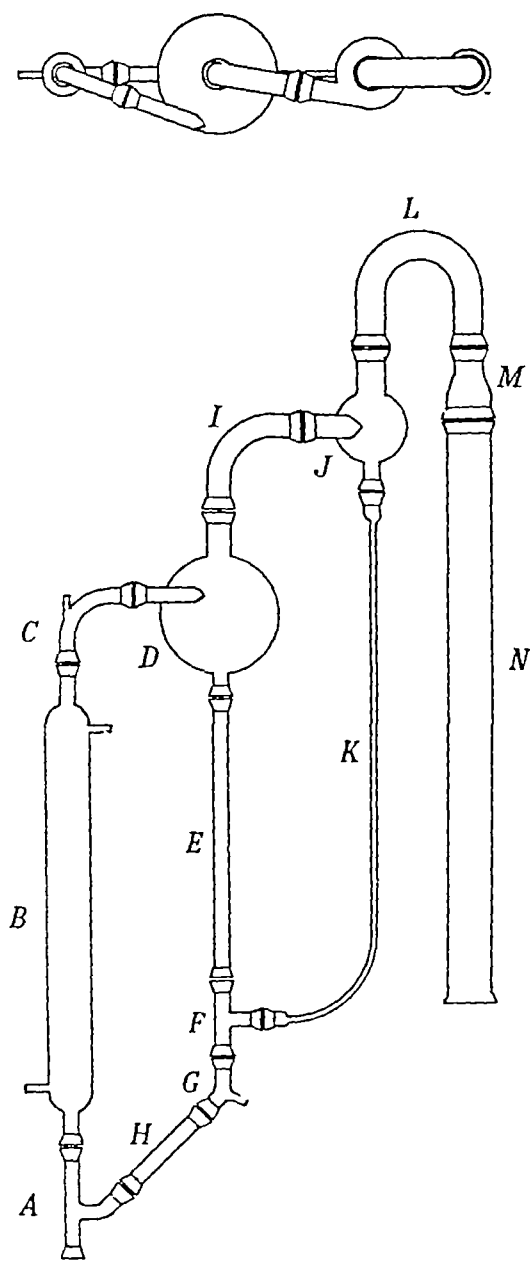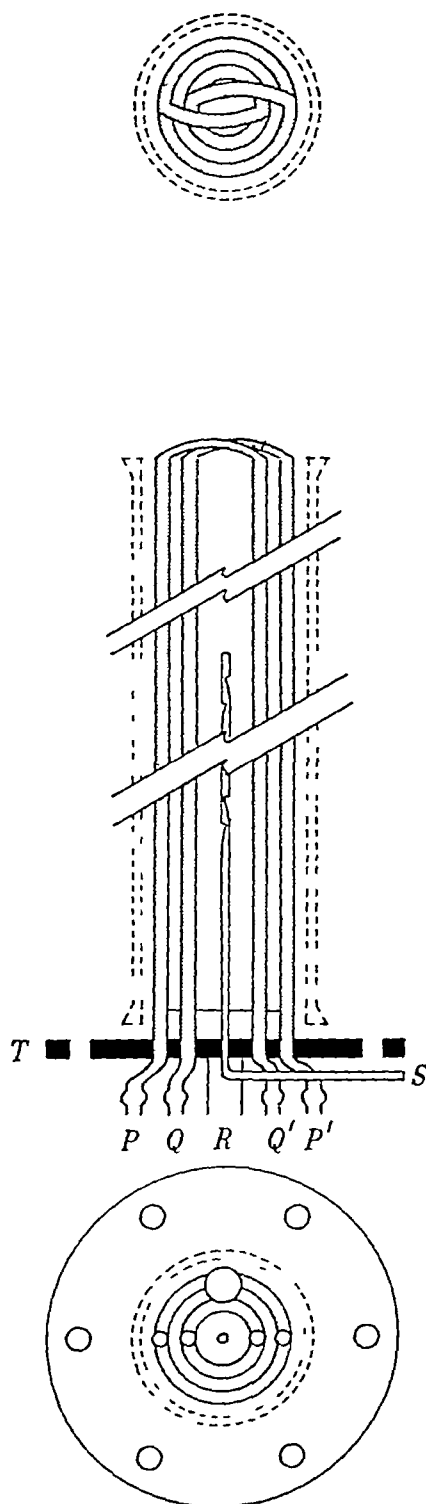
Fig 1

Fig 2

arranged concentrically to give four cooling surfaces with a combined area of 920 sq in The top and bottom ends of adjacent pairs of tubes are turned towards each other and welded together to form two hollow-walled cylinders which are connected together at the top, as shown, by two welded-in lengths of $\frac{1}{4}$ in copper tubing At the bottom ends the hollow walls of these cylinders are connected to the water supply by $\frac{1}{4}$ in copper Tubes PP' and to the drain by Tubes QQ' Cold water can be passed through the hollow walls of the two cylinders at a considerable rate and very efficient condensing can thus be attained These tubes, and Tubes R and S, pass through, and are welded on to, a $6\frac{1}{2}$ in diameter $\frac{1}{4}$ in brass Disc T

R is a $\frac{1}{2}$ in brass tube connected by means of a Y-piece to two efficient water jet pumps The condensate is discharged via this tube and the pumps into the drain S is a $\frac{3}{16}$ in copper tube reaching about 18 in into the lumen of the condenser , it is perforated near the top at the condenser end, and at the other end it is connected to a mercury manometer and a vacuum release valve A rubber interface joint gasket is placed between the end of N and the top of T, and these two parts are bolted together in the usual way

A well-fitting rubber bung is inserted in the bottom of the Y-piece A, and through this bung passes the upper limb of a + piece of $\frac{5}{16}$ in glass tubing The lower limb and the left-hand limb are fitted with $\frac{1}{4}$ in taps, and the right-hand limb is connected by means of a heavy wall capillary tube fitted with a tap to a reservoir of antifrothing agent The liquid to be concentrated enters the apparatus through the tap in the left-hand limb, and the concentrate is discharged through the tap in the bottom limb Before passing into the apparatus the dilute liquor passes through a " Rotameter " flow-meter calibrated for water flowing at rates from 6–16 l /hr

Operation

The water jet pumps are turned on and the dilute liquor is drawn up into the apparatus to a level about 3 in below the bottom of Bowl D The inlet tap is then turned off, the pressure is brought down to 15–20 mm Hg , and steam issuing from a valve at about 7 lb pressure is allowed to pass freely through the inner and outer jackets of the heat exchanger B The liquid in B immediately starts distilling, and the vapour thus formed forces liquid into Bowl D at great speed Here it is subjected to centrifugal force which separates the vapour from the liquid , the latter returns to the heat exchanger via $E\,F\,G\,H\,A$ while the vapour passes to the second centrifugal separator J, where any residual liquor is returned to the heat exchanger through K From J the vapour passes through L to be rapidly condensed in N After about a minute the inlet tap is opened enough to allow the dilute liquor to enter the apparatus at about the same rate as water is being distilled off With tapwater at about 15° C the rate of distillation is 12–14 l /hr , and the temperature of the liquid being distilled (as measured with the thermometer in the elbow C) does not rise above 36° C. It is important to judge the correct rate of inflow of the dilute liquor, and for this purpose the flowmeter is a great advantage If the dilute liquor is fed in too quickly the rapidly rotating current of water vapour will carry liquid up into the elbow I and constrict it and thus slow down the rate of evaporation If the dilute liquor is fed in too slowly for a long time there is danger that the con-

centrate will dry in the heat exchanger When the apparatus was first constructed the parts E and F were reversed so that the return arm K came in at a higher level, as it does in the apparatus of Mitchell *et al* (1944) With this arrangement there was not a sufficient hydrostatic head to keep an unbroken column of liquid in K The result of this was that vapour rushed through K, carrying liquid with it and splashing it up into L, whence it was carried over to the condenser by the stream of water vapour This led to considerable " creep " loss With the present arrangement no such difficulty is met

The vapour from D enters J at tremendous speed—about 150 1 per sec —which means that it passes through the 1$\frac{1}{2}$ in elbow I at 280 miles per hour The effect of this was seen when, for reasons now irrelevant, a heavy glass stopper was placed in the bottom of Bowl J As soon as distillation started the stream of vapour lifted up the stopper and whirled it around at great velocity for a fraction of a second before hurling it through the side of the bowl

The centrifugal action to which the distilling liquid is subjected in Bowl D breaks down considerable froth, but with very violently frothing liquids it is advisable to introduce 0 5 ml of an anti-frothing agent, preferably a non-volatile one such as triamylcitrate, at the beginning of the distillation

A given volume of liquid can be concentrated down to about 750 ml provided the concentration of total solids is not high enough to render the concentrate unmanageably viscous When dealing with volumes of the order of 100 1 (*ca* 0 2 per cent total solids), or with liquids containing high concentrations of solids, it might be advisable continuously to draw off the concentrate through the side tube on S This could be done by attaching a tap and a flask evacuated to a higher degree than the rest of the apparatus This procedure has not been tried out by the author It is probably more convenient to stop the distillation at suitable intervals and draw off the concentrate

Distillation is stopped by first turning off the steam , after about five minutes, when the heat exchanger has cooled down, the vacuum is released, the water jet pumps are turned off and the concentrate is drawn off The apparatus can be cleaned as follows Draw in water up to about half-way in Bowl D and reduce the pressure to about 60 mm Hg Then open the inlet tap This results in a violent swirling motion of the wash water, which scours the walls of the apparatus This is repeated several times with changes of water, and, if necessary, with hot water or with dilute acid or alkali or a detergent Finally, fresh water is sucked through the entire apparatus for some time and allowed to discharge through the water jet pumps

SUMMARY

The construction and operation of a laboratory circulating evaporator which is capable of evaporating water at the rate of 12–14 1 /hr at 34–36° C and 20–30 mm Hg is described

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ANTIBIOTICS FROM A STRAIN OF *B SUBTILIS* BACILIPIN A AND B AND BACILYSIN

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THE production of a number of antibiotics has already been attributed to strains of *B subtilis*, but since there is no universally accepted classification for this organism all workers may not have used the same species of aerobic spore-forming bacillus   One method of classification in present use is given by Gibson and Topping (1938) and Gibson (1945), and another by Smith, Gordon and Clarke (1946)

Two of these antibiotics, subtilin (Jansen and Hirshmann, 1944) and bacitracin (Johnson, Anker and Meleney, 1945) have been obtained in a relatively pure condition and shown to be polypeptides   The remainder have been less well defined and some may be identical   Among them, bacilysin (Gilliver, Abraham and Florey, 1946, unpublished, Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949), bacillin (Foster and Woodruff, 1946) and subtenolin (Hirschhorn, Bucca and Thayer, 1948) are probably peptides, while endosubtilysin (Olivier, de Saint-Rat, Bonét-Maury and Blanchon, 1945), subtilysine (Vallée, 1945a, 1945b) and eumycin (Johnson and Burdon, 1947) are probably organic acids

The organism used in the present investigation (A14) was isolated by Miss M Savage from a contaminated culture of *Myco tuberculosis* brought from Chile by Dr A Arriagada   It was identified as a strain of *B subtilis* by Miss K Gilliver, using the method of Gibson and Topping (1938)   On solid medium the growth of various bacteria, inoculated at right angles to a streak of the organism which had grown for 24 hours at 37° C, was inhibited to distances given in Column 1 of Table I

This paper records the results of an investigation of the antibacterial products formed by the strain of *B subtilis* A14 in aerated liquid media   Under the conditions used the organism produced at least three kinds of antibiotic

MEDIUM AND CONDITIONS OF CULTURE OF THE ORGANISM

The organism was grown in the salts of Czapek-Dox medium with the addition of 0 5 per cent neutral corn steep liquor and 0 5 per cent glucose   The medium was made up with tap water

Antibacterial activity was measured by the cylinder plate method (Heatley, 1944), using *Myco phlei* and *Staph aureus* (H strain N C T C No 6571) as test organisms

When the organism was grown in stationary shallow layers at 37° C , the culture fluid showed no activity against either test organism   Aeration of the culture medium favoured antibiotic production   The most convenient temperature for growing aerated cultures was 34° C   At this temperature maximum activity was produced after twelve-hour growth from a 2 to 3 per cent inoculum of a nine-hour aerated culture

The amount and quality of aeration was found to control the extent to which the antibacterial range of the culture fluid reproduced that found in the streak test   When aeration rates were low, or the bubble-size coarse, the culture fluid showed activity against *Myco phlei* only   As the rate and efficiency of aeration was increased, activity against *Staph aureus*, corynebacteria and gram-negative organisms was also noted, and this increased until an *optimum aeration* was reached   The actual amounts of air required varied with the shape and size of the culture vessel and the type of diffuser   Using glass bottles 4 in in diameter containing one litre of culture fluid and an aloxite aerator stone 2 cm × 2 cm × 5 cm (Carborundum Co) the threshold amount to produce activity detectable by the cylinder-plate method against organisms other than *Myco phlei* was two volumes per volume of culture fluid per minute (i e 2 1 per minute in these vessels)   The optimum was reached at ten volumes per volume per minute   These very high rates could be reduced to one volume per volume per minute, and still give maximum production, in twenty-litre vessels 12 in in diameter, when diffusers covering nearly the whole of the bottom of the vessel were used   The changes in pH and the percentage of the final activity produced at hourly intervals is shown in Fig 1   Spores began to appear at 14 hours and by 20 hours only a few vegetative organisms remained
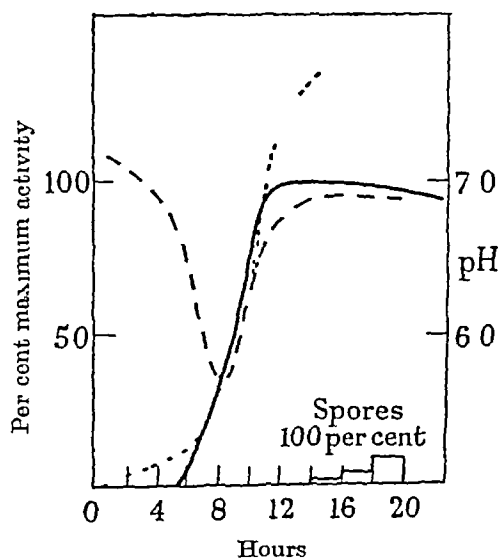

Fig 1 —Activity and pH changes during aerated culture of *B subtilis* (A 14) 34° C — Per cent maximum activity against *Staph aureus* and *Myco phlei* for both types of vessel described ,   per cent maximum activity *Myco phlei* only , — —pH 11 vessels 10 vol air/vol /min , — pH, 20 l vessels 1 vol air/vol /min

The antibacterial range of the culture fluid after 12 hours' incubation under conditions of optimum aeration is shown in Column 2, Table I

TABLE I

Organism	(1) Length in mm of inhibition of growth of secondary streak	(2) Length in mm of inhibition produced on a wheel-plate by aerated culture fluid
Myco phlei (Lab)	14	15
Staph aureus N C T C No 6571	(11)	10*
C diphtheriae var *gravis*	6	8
C xerosis	4	8
Salm enteritidis Gaertner	(10)	6
Salm typhi	2	5
Streptococcus	0	0
Bact coli	0	2
Ps pyocyanea	0	0

( ) Partial inhibition only   * Some resistant colonies

NATURE OF THE DIFFERENT ANTIBIOTICS

The culture fluid contained three distinct groups of antibiotics

Group I

Two acidic substances which were readily extracted into organic solvents, other than petroleum ether, at pH 5 0, and which accounted for 75 per cent of the activity against *Myco phlei* The names bacilipin A and bacilipin B have been given to these substances

Group II

A stable peptide which remained in the culture fluid after extraction of the bacilipins This was similar to, and was possibly identical with, an antibiotic mentioned by Abraham, Callow and Gilliver (1946) which has been called bacilysin (Gilliver, Abraham and Florey, 1946, unpublished) Bacilysin accounts for almost all of the activity of the culture fluid against *Staph aureus*, and the coryne-bacteria, and for 25 per cent of the activity against *Myco phlei*

Group III.

An unstable substance (or substances) responsible for the activity of the culture fluid against gram negative organisms Its activity in the harvested culture fluid was considerably reduced after 24 hours at 4° C , but it was more stable after heating the fluid at 70° C for ten minutes Possibly an enzyme system produced by the organism was responsible for its destruction This group has so far been little investigated

Bacilipin A and B.

Assay

The cylinder-plate method of assay was used throughout the purification *Staph aureus* grown on heart-agar was used as a test organism Although the

bacilipins only produce zones of partial inhibition when tested against *Staph aureus*, this organism was found to give a more reliable assay than *Myco phlei*

Purification

Extraction from culture fluid —Bacilipins A and B are extracted quantitatively from the culture fluid, at pH 5 0, into a quarter of a volume of amyl acetate Since back extraction into water was not quantitative and distillation produced considerable inactivation, the activity in the amyl acetate was concentrated on alumina by percolation through four columns in series In this way the active material from one litre of amyl acetate concentrate could be adsorbed on 10 ml of Brockmann alumina pH 9 0 An eluting mixture of one-fortieth of a volume of M/2 potassium phosphate just saturated with acetone was then run through the columns in series The elution of the activity was not found to be correlated with any coloured bands The combined eluates contained from 30 to 50 per cent of the activity originally in the amyl acetate , the remainder of the activity left on the columns tailed too much to warrant efficient recovery The bacilipins were then extracted into ether, which was concentrated to a convenient volume *in vacuo* and further purified by the counter-current distribution method (Craig, Golumbic, Mighton and Titus, 1945) Early counter-current studies revealed the presence of two active substances, A and B (Fig 2)
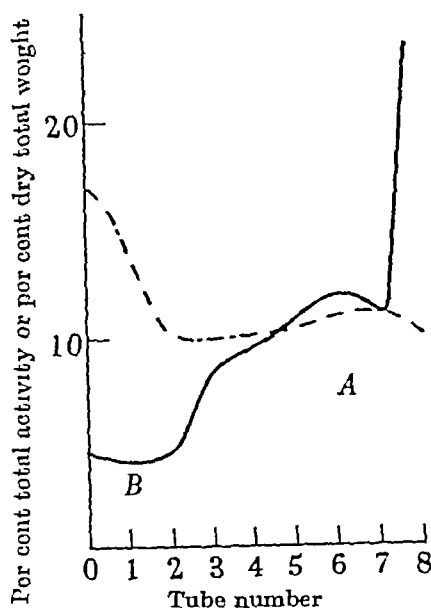

Fig 2 —Counter-current distribution pattern of a mixture of bacilipins A and B pH 6 8,

$$K \text{ (mixture)} \quad \frac{\text{(Ether)}}{\text{(M/2 Buffer)}} = 1 \ 0 \text{ approx},$$

Buffer mobile
— —, per cent total activity , —, per cent dry weight

Separation of bacilipin A and B —Titration curves for the transfer of the separated bacilipin A and B from ethereal to aqueous solution (Fig 3) showed subsequently that A and B could be separated by fractional partition between ether and buffer solutions (Table II) This enabled the initial counter-current distribution to be dispensed with
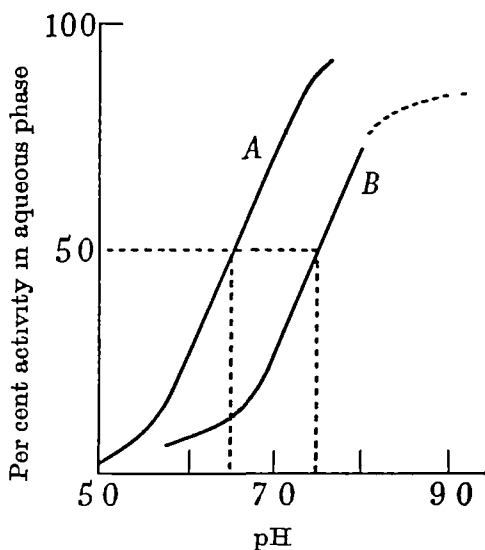
FIG 3 —Distribution curves for bacilipins A and B between ether and M/2 potassium phosphate

Counter-current distribution —The separated fractions were each purified further by successive ten or twelve counter-current distributions in separating funnels   Ether was used as the static and M/2 potassium phosphate buffer as the mobile phase   The partition coefficients were approximately one when the buffer solution was at pH 6 5 for A and at 7 8 for B   Very stable emulsions were produced with this system unless the concentration of the crude substance was kept below 4 mg /ml
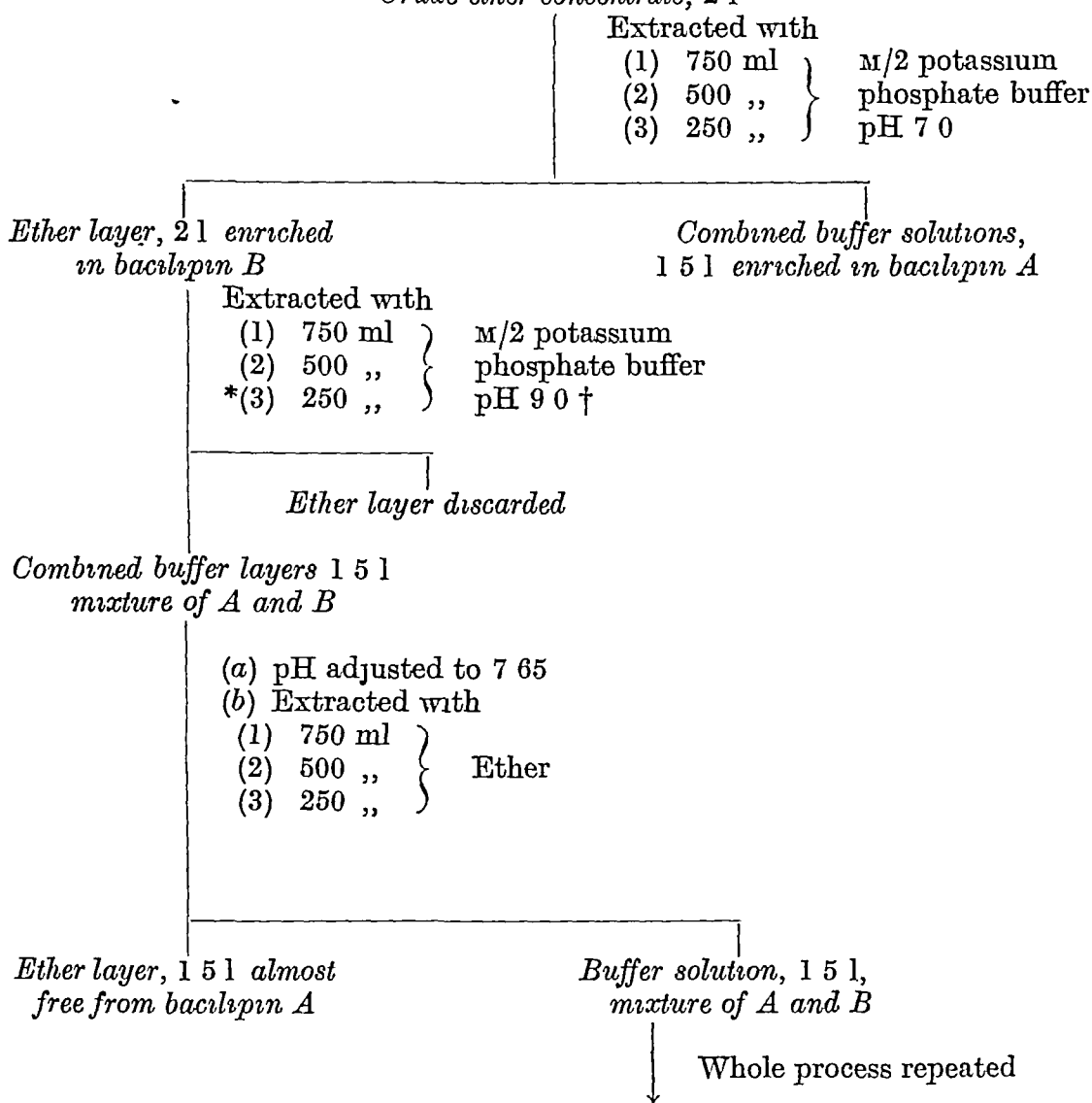
The necessity of using dilute solutions of the active substances made the use of Craig's stainless steel apparatus (Craig, 1944) quite impracticable for work on a preparative scale, thus rendering the procedure more laborious and less efficient

At the end of each run all the material in each tube was transferred to the ether layers, which were analysed to give the percentage of the total dry weight and total activity present in each   The method of selecting " strategic fractions " (Craig, 1944) was then used   The strategic fractions were pooled and further runs carried out, until some of the material behaved as though it was homogeneous, in that the partition coefficient remained constant over a number of tubes (Williamson and Craig, 1947)

Fig 4 shows the first, second and third runs on " B " and Fig 5 the first and fourth runs for " A "   From two hundred titres of culture fluid 110 mg of A and 60 mg of B, in ethereal solutions, were shown to be practically homogeneous within the limits of resolution of the method

Chemical properties

Attempted isolation in the solid state —On drying solutions of the free acids A and B in organic solvents, or solutions of their salts in water, an irreversible reaction occurred, even at low temperatures   From 50 to 70 per cent of the activity was lost initially and the loss increased further on standing   The loss of activity was accompanied by a decrease in the amount of hydrogen absorbed on catalytic hydrogenation and by a sharp decrease in solubility in organic solvents

TABLE II —*Separation of Bacilipin A and B*

Crude ether concentrate, 2 1

Extracted with
(1) 750 ml ⎫ M/2 potassium
(2) 500 ,, ⎬ phosphate buffer
(3) 250 ,, ⎭ pH 7 0

Ether layer, 2 1 enriched   *Combined buffer solutions,*
in bacilipin B   *1 5 1 enriched in bacilipin A*

Extracted with
(1) 750 ml ⎫ M/2 potassium
(2) 500 ,, ⎬ phosphate buffer
*(3) 250 ,, ⎭ pH 9 0 †

                    Ether layer discarded

Combined buffer layers 1 5 1
mixture of A and B

(a) pH adjusted to 7 65
(b) Extracted with
    (1) 750 ml ⎫
    (2) 500 ,, ⎬ Ether
    (3) 250 ,, ⎭

Ether layer, 1 5 1 almost   *Buffer solution, 1 5 1,*
free from bacilipin A   *mixture of A and B*

                                               Whole process repeated

* Ether layer evaporated to 250 ml for this extraction
† Boric acid interferes when borate buffer is used

Elementary analysis —Elementary analyses of the lyophil-dried barium salts (Weiler and Strauss, Oxford) gave the following results

A C, 42 6 , H, 6 3 , N, 2 5 , Ba, 24 6

B C, 52 45 , H, 6 75 , N, 2 09 , Ba, 21 6

Sulphur and phosphorous were absent

Since these values are for the dried substances they may differ significantly from those for the fully active barium salts Lack of material prevented an attempt to deduce the elementary composition of the active substances from analyses carried out after hydrogenation
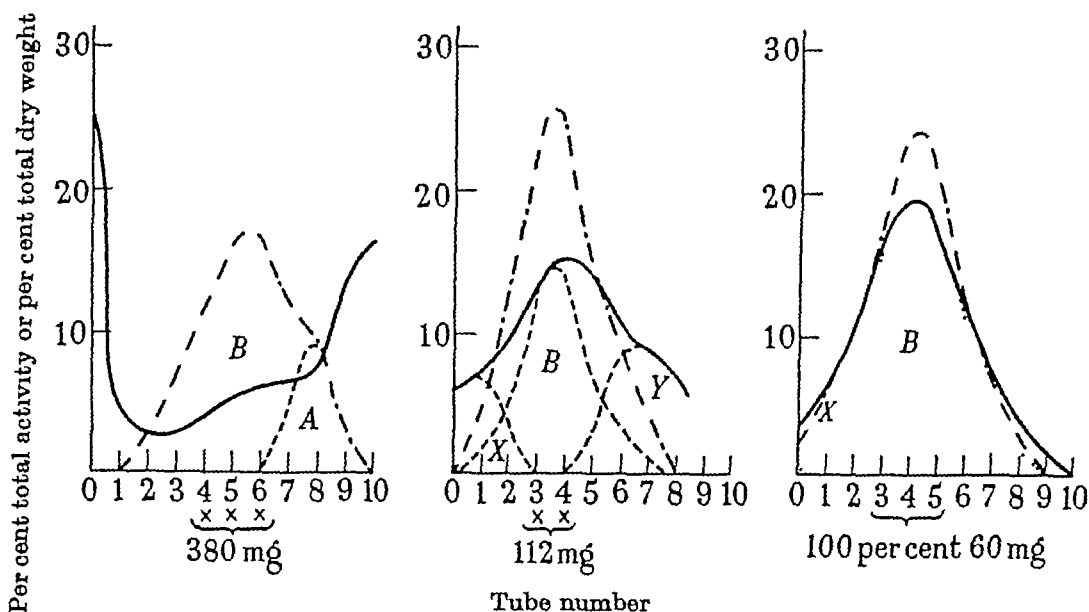
FIG 4 —Three successive ten tube counter current distributions on 2 5 g crude bacilipin B, showing strategic fractions selected. pH 7 9, $K = \frac{\text{(Ether)}}{\text{(M/2 Buffer)}} = 1\ 4$, the aqueous phase was mobile

— —, per cent total activity, — per cent dry weight, , theoretical curve $K = 1\ 4$, — — —, approximate theoretical interpretation, xx, strategic fractions selected

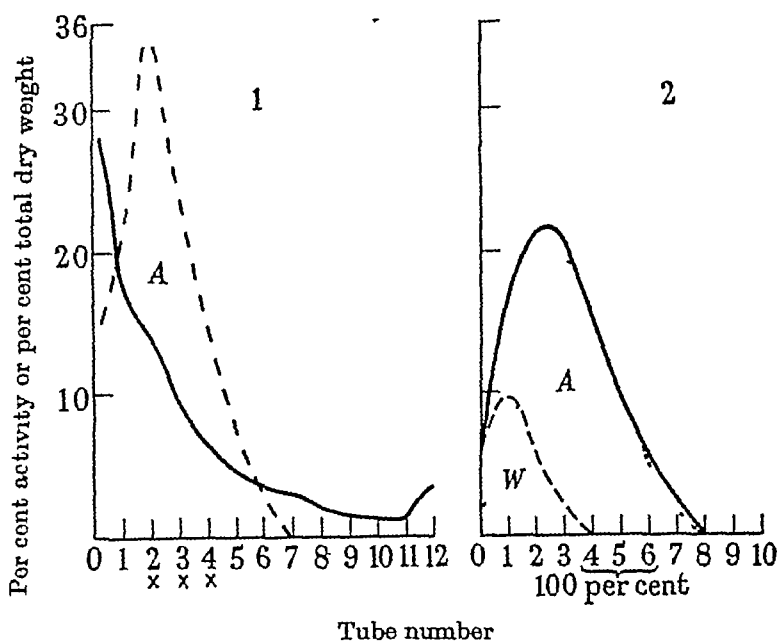FIG 5 —(1) Initial twelve tube distribution on crude bacilipin A.

pH 6 0, $K = \frac{\text{(Ether)}}{\text{(M Buffer)}} = 3\ 3$ approx

(2) Fourth run, pH 6 5, $K = \frac{\text{(Ether)}}{\text{(M/2 Buffer)}} = 1\cdot7$, derived curve W is almost that for a homogeneous substance $K = 6\ 7$

— —, per cent total activity, —, per cent dry weight, , theoretical curve $K = 1\ 7$, — — —, (per cent total dry weight, minus theoretical curve A), xxx, strategic fraction selected.

The equivalent weight of the dry barium salt of A was 278 and that of B was 317 5 Since the ratio Ba/N was one this indicated that the minimum molecular weight of A was 556 and that of B was 635

Solubility and general properties —The free acids A and B were readily soluble in amyl acetate, benzene, chloroform and ether, but insoluble in petroleum ether They had a very limited solubility in water, being precipitated as unstable oils on adjusting a neutral aqueous solution to pH 3 0 The sodium and barium salts were soluble in water and also in absolute alcohol

Both A and B gave a negative Molish reaction, formed no precipitate with 2 4-dinitrophenylhydrazine and did not reduce ammoniacal silver nitrate

Both substances readily absorbed bromine in chloroform solution The bromo compounds were dark viscous oils and were biologically inactive

Acid base properties —Titrations made with a glass electrode showed that A was a stronger acid than B (Fig 6) The titration curves showed no indication of the presence of a basic group

After drying over phosphorous pentoxide the equivalent weight of A, found by titration to pH 7 6, was nearly double that of the undried substance The original value of the equivalent weight was obtained, however, when further alkali was added and the solution was warmed This suggested that lactonization had taken place

Hydrogenation —Hydrogenation of A and B in a Warburg manometric apparatus with an Adams or palladium/barium sulphate catalyst showed that the degree of unsaturation of the substances was similar but decreased after drying from aqueous solutions Thus hydrogenation of the barium salt of A in aqueous solution gave the following figures for hydrogen uptake

Before drying 180 c mm /mg

After drying 68 c mm /mg

The dry barium salt had lost 75 per cent of the activity shown by the solution The difference in both hydrogen uptake and activity was even greater after the solid had been kept for a few days

An oil was obtained when a solution of the hydrogenated sodium salt of A (undried) was acidified to pH 2 0 This crystallized in sheaves of needles m p 78° to 76° C when the turbid solution was left in contact with petrol ether overnight Extraction of the supernatant with ether yielded an oil mixed with some crystals of m p 95° to 100° C The amounts of crystalline material available were insufficient for further investigation Only oily material was obtained when hydrogenated B was treated in a similar manner.

Stability in solution —In amyl acetate 25 per cent of the activity of a dilute solution of the free bacilipins A and B was lost after heating for one hour at 70° C

Aqueous solutions of salts of the bacilipins were stable within the range pH 2 0—pH 10 0 at 16° C for four hours but were rapidly destroyed at 100° C At pH 13 inactivation of A and B occurred at room temperature Subsequent electrometric titration indicated that the inactivation was accompanied by the liberation of a basic group and two new acid groups (Fig 6) The products of inactivation did not give a ninhydrin reaction

The activity of bacilipin A and B was not reduced by incubation with 50 per cent serum or with trypsin at pH 8

Acid hydrolysis —When 2 mg of the barium salts of both A and B were heated with 6N hydrochloric acid in a sealed tube at 100° C overnight, much " humin " was produced by B but considerably less by A Extraction of the hydrolysates with ether and evaporation of the extract yielded an oil from A and crystals m p 105° C from B

The ether-insoluble material resulting from the hydrolysis of both A and B gave a positive ninhydrin reaction The colour yield from A was about half, and from B a quarter, of that given by a standard solution of glycine containing an equivalent amount of nitrogen

Paper strip chromatograms (Consden, Gordon and Martin, 1944), using phenol-5N hydrochloric acid, were run with 5 μl (= 5 γN) of a solution of the hydrolysates of A and B but no spots were revealed on treatment with ninhydrin After hydrolysis about one-fifth of the total nitrogen was present as volatile base, and this was probably responsible for the ninhydrin reaction
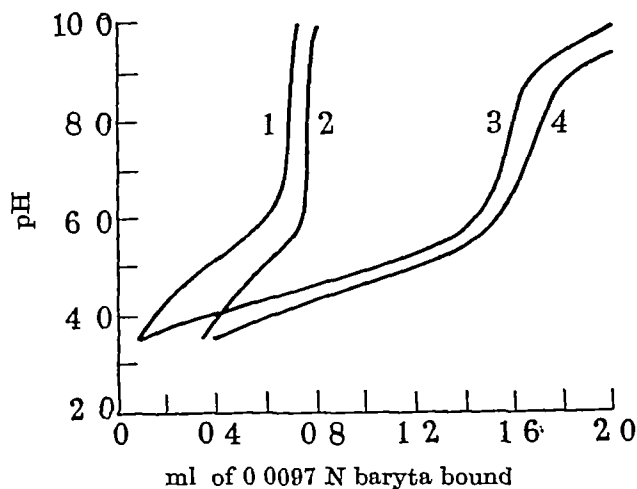

Fig 6 —Electrometric titrations on 2 04 mg bacilipin A and B before and after inactivation at pH 13 0 Curve (1), active bacilipin B , Curve (3), inactive bacilipin B , Curve (2), active bacilipin A , Curve (4), inactive bacilipin A

Discussion

Bacilipins A and B appear to be similar and to be chemically related The limited chemical information that could be obtained from the small amounts of material available indicates that the bacilipins are nitrogenous unsaturated aliphatic dibasic acids Since two acid groups and a basic group are liberated by M/10 sodium hydroxide the substances probably contain an amide type of linkage The base does not give a ninhydrin reaction and is not volatile The other acid group liberated may arise from the hydrolysis of an ester linkage or the opening of a lactone ring

However, the results of a single elementary analysis of the dried barium salts of both bacilipin A and B appeared to reveal a degree of saturation which does not allow all these groupings to be fitted into the molecule and also account for the observed hydrogen uptake of the active substances on catalytic hydrogenation It is possible that the barium salts are still hydrated after drying and that the inactivation which occurs on drying involves oxidation across the double bonds

Since the antibacterial properties of the compounds did not justify the considerable amount of work which would have been required to obtain larger amounts of the pure substances, further investigations on bacilipin A and B have been discontinued

Bacilysin

Partial purification

Bacilysin was assayed by the cylinder-plate method (Heatley, 1944) throughout the purification *Staph aureus* grown on heart agar was used as the test organism

The culture fluid was acidified to pH 3 0 and clarified in a Sharples centrifuge The pH was then adjusted to 2 0 and 1 5 per cent Farnell phosphoric-activated carbon was added The mixture was agitated for twenty minutes and then filtered The bacilysin was eluted by two extractions with a quarter volume of a mixture of four volumes of ethanol and one of м/2 potassium phosphate pH 7 0 This amount of phosphate buffer is just sufficient to saturate the alcohol phase and leave a small volume, about 50 ml per litre, of the aqueous phase Only the alcohol phase appears in the eluates Overall recovery under favourable conditions has been 70 per cent

The alcoholic eluate was percolated rapidly through a column of Brockman alumina pH 5 0 until the column was nearly saturated (300 ml per 10 ml alumina) Bacilysin was readily eluted from the alumina by raising the water content of the solvent Elution with 25 per cent ethanol was found to give material of the highest activity There appeared to be no correlation between bands visible under U V light and the active material A standardized ninhydrin colour reaction carried out on aliquots of the effluent fractions, showed that inactive peptides or amino acids continued to be eluted after the bacilysin had been removed from the column Bacilysin was obtained as an amorphous white powder when the eluates were distilled to a small volume *in vacuo* and the aqueous residue lyophil-dried Material obtained in this way from the most active " cut " from the columns was used in some preliminary chemical investigations

Chemical properties

The preparation of bacilysin contained nitrogen and sulphur It was practically insoluble in all dry organic solvents except methanol It was readily soluble in water, and its solubility was not appreciably reduced by saturation with ammonium sulphate An aqueous solution formed no precipitate with picric acid, phosphotungstic acid, trichloroacetic acid or basic lead acetate It gave positive ninhydrin and Pauly diazo reactions The Molish test was negative

Stability

Bacilysin was stable for 4 hours at 20° C between pH 1 4 and 12 0 It was not destroyed when heated to 100° C for fifteen minutes at pH 7 0 to 9 4 However, when heated for ten minutes at pH 1 0 over 90 per cent of the activity was lost

22

Incubation with crystalline trypsin and with tissue enzymes at pH 8 0 rapidly inactivated bacilysin   Treatment with hydrogen sulphide reduced the activity by half

Hydrolysis and paper chromatography

Paper chromatograms after hydrolysis with 6 N HCl at 100° C showed that the material contained alanine, tyrosine, leucine and phenylalanine

The evidence at present available indicates that bacilysin is a neutral peptide

ANTIBACTERIAL PROPERTIES OF BACILIPIN A AND B AND BACILYSIN

Bacilipins A and B

Cylinder-plate test —Assay by the cylinder-plate method using *Staph aureus* and *Myco phlei* as test organisms gave zones of inhibition 20 to 25 mm in diameter at the following dilutions

	Staph aureus	*Myco phlei*
A	1/10,000	1/100,000
B	1/80,000	1/800,000

The plates were seeded with a 12-hour broth culture of *Myco phlei*, and with a 12-hour culture diluted 1 100 of *Staph aureus*   They were read after 12-hour incubation

The inhibition was only partial with *Staph aureus* but remained constant on further incubation   With *Myco phlei* the zones of inhibition were clear but were much reduced after 48 hours' incubation

Wheel-plate test —When tested by the wheel-plate method at 1/1000 concentration against various bacteria, the lengths of inhibition shown after 15-hour growth by the susceptible organisms were as follows

	Staph aureus	*Myco phlei*	*B anthracis*
A	(7 mm)	15 mm	5 mm
B	(15 „)	20 „	12 „

( ) partial inhibition

Bact coli, Salm enteritidis Gaertner, *C xerosis, Strep pyogenes* strain, Kruger, *C diphtheriae* var *gravis* and *Ps pyocyanea* were not inhibited

Serial dilution test —Serial dilutions using *Myco phlei* as test organism made with an impure mixture of A and B of unknown relative composition gave an endpoint of 1/320,000 when read at 15 hours' growth, but this decreased to zero after 48 hours   Pure B was later shown to be about twenty times more active by the cylinder-plate method than the sample used in this test

Partially-purified bacilysin

Wheel-plate test —When tested by the wheel-plate method at a concentration of 1/1000, bacilysin inhibited the growth of various organisms to the following distances from the cylinder

Bact coli	*Staph aureus*	*C xerosis*	*Myco phlei*
(5 mm)	10 mm	10 mm	(5 mm)

The inhibition of *Bact coli* and *Myco phlei* was only partial and decreased after 38 hours' incubation   A few resistant colonies were found within the zone of inhibition against *Staph aureus* (Abraham, Callow and Gilliver, 1946) *B anthracis* and *Strep pyogenes* were not inhibited

Combined action of bacilysin and the bacilipins

Cylinder-plate test —When the culture fluid was tested by the cylinder-plate method against *Staph aureus* or *Myco phlei*, the zones of inhibition were clear, and remained constant in diameter if the period of incubation was extended from 18 to 48 hours   In contrast, the pure bacilipins did not produce a permanent zone of inhibition when tested against *Myco phlei*, the zones gradually diminishing in size through growth at the circumference, and when bacilysin was tested against *Staph aureus*, resistant colonies were found within the zone of inhibition A reconstructed mixture having similar concentrations of the antibiotics to those in the culture fluid gave the same type of inhibition as that of the culture fluid itself (Fig 7)

Serial dilution method —Serial dilution tests were made with bacilysin alone and with a mixture of bacilysin and bacilipin A (10 1), using *Staph aureus* as the test organism   In both cases visible growth was inhibited after 16 hours incubation when the concentration of bacilysin was 1 in 320,000   On further incubation all the tubes containing bacilysin alone eventually showed the same turbidity as the control, but in the tubes containing the mixture of antibiotics further growth was considerably retarded and inhibition was still complete at 1 in 20,000 after 4 days

Conclusion

It appears that the bacilipins are bacteriostatic towards *Myco phlei*, for while they initially inhibit growth at high dilutions multiplication finally occurs and the organisms in the resulting culture have no increased resistance   Bacilysin delays visible growth of *Staph aureus* in high dilutions, but in this case there is also eventual multiplication   The effect of bacilysin is due to a partial bactericidal action followed by the growth of resistant surviving cells (Abraham, Callow and Gilliver, 1946)

Bacilysin and the bacilipins combined are considerably more effective in suppressing bacterial growth than is either alone   This accounts for the fact that the crude culture fluid produces clearer or more permanent zones of inhibition than the purified antibiotics when tested by the cylinder-plate method

RELATIONSHIP OF THE BACILIPINS AND BACILYSIN TO OTHER ANTIBIOTICS FROM *B subtilis*

Bacilipins —Other organic acids so far obtained from *B subtilis* appear to be distinct from the bacilipins   Thus, endosubtilysin (Olivier, de Saint-Rat, Bonét-Maury and Blanchon, 1945) is insoluble in acetone   Subtilysine (Vallée, 1945a, 1945b) is insoluble in 10 per cent calcium chloride and eumycin (Johnson and Burdon, 1946) is insoluble in ether and amyl acetate

The active principle " M and R " (Miller and Rowley, 1948) obtained from a *B mesentericus* shows similarities to the bacilipins in its general chemical pro-

perties However, the most purified sample of M and R contains at least twice as much nitrogen as the bacilipins and is active against a much wider range of organisms It would appear that strains of *B subtilis* produce a number of organic acids having antibacterial properties, in addition to several biologically active peptides

Bacilysin —Among the antibacterial peptides produced by strains of *B subtilis* are bacitracin, subtenolin, subtilin and bacillin The fact that bacitracin and subtenolin form a relatively insoluble picrate distinguishes them from bacilysin and also from subtilin and bacillin Of the latter three substances, subtilin, unlike bacilysin, is readily salted out from aqueous solution and contains at least twelve different amino acids, while bacillin is equally active against gram-positive and negative organisms, when tested in nutrient agar Bacilysin, therefore, appears to differ from other antibacterial peptides hitherto described

SUMMARY

A strain of *B subtilis* A14 produces four different antibiotics when grown in submerged aerated culture in a modified Czapek-Dox medium Two of the antibiotics can be extracted by organic solvents and have been named bacilipin A and bacilipin B A third antibiotic is insoluble in organic solvents and is called bacilysin The fourth substance differs from the other three in that it is active against *Bact coli* and is rapidly destroyed in the harvested culture medium

Bacilipin A and bacilipin B are unsaturated aliphatic nitrogenous acids and have similar properties They are stable in solution at room temperature but have only been isolated in the solid state with loss of activity

Bacilysin is a water-soluble neutral peptide which appears to be of relatively simple composition The most active preparation yields alanine, tyrosine, phenylalanine and leucine on hydrolysis Bacilysin is inactivated by trypsin and tissue enzymes

The bacilipins are active mainly against *Myco phlei* and their action appears to be bacteriostatic Bacilysin is active against *Staph aureus, C xerosis, Myco phlei* and to a lesser extent *Bact coli* Its action is mainly bactericidal However, when tested against *Staph aureus* some organisms survive and these are found to be resistant to bacilysin after subculturing Mixtures of the bacilipins and bacilysin show a combined action, which is more effective in suppressing bacterial growth than is either component when tested alone

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CONSDEN, R , GORDON, A H , AND MARTIN, A J P —(1944) *Biochem J* , **38**, 224
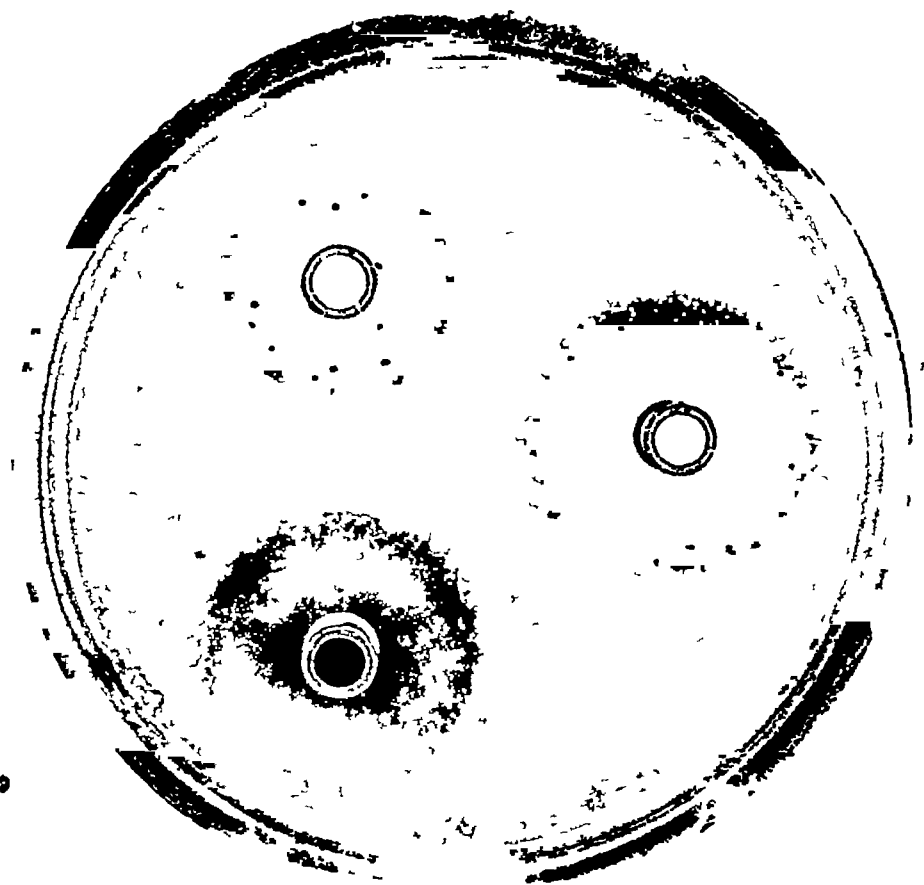CRAIG, L C —(1944) *J biol Chem* , **155**, 519

FIG 7 —Combined action of bacilysin and bacilipin B against *Staph aureus* Top left zone, bacilysin 1/4000 showing resistant colonies , bottom left, bacilipin B 1/10,000 showing partial inhibition only , right, a mixture containing bacilysin 1/4000 and bacilipin B 1/10,000 showing a zone of complete inhibition

Newton

injection, and this determined the neutralization of intradermal toxin at the time of its injection into the skin These conclusions were in accord with the fact that when antitoxin was given *after* the toxin, its efficacy was greatly diminished, even when the toxin lesions were only a few minutes old They also measured the amount of antitoxin neutralizing toxin *in vitro* (the *direct* neutralizing quantity, symbolized by Ad) and *in vivo* after intravenous injection (the *indirect* neutralizing quantity, symbolized by Ai) and found the ratio Ai Ad to be 22,500 1 Allowing for the plasma volume of the rabbit and the volume of toxin injected, and assuming that tissues and antitoxin did not compete for the toxin, they deduced a ratio of 22 5 1 for the relative concentration of antitoxin in the circulation and the tissues

The concepts of a rapidly established concentration of antibody in the tissues, fully effective (i e *definitive*) in the sense that when toxin is injected, no antibody subsequently passing into the tissues from the blood adds to the neutralizing effect , and of a rapid and irreversible fixation of toxin to the tissues in the absence of antibody, have a direct bearing on passive antibody therapy , and were accordingly re-examined

MATERIALS AND METHODS

One batch of diphtheria toxin and one of antitoxin were used throughout. The toxin was prepared by Dr L Holt on a semi-defined medium , it contained 0 1 per cent total N and 78 Lf per ml , at Lf 52 its Kf was 10 It was preserved at 2° C under toluene The antitoxin was a commercial preparation of proteolytically refined horse serum containing 4000 international units (u) per ml , standard stock dilutions were made in 50 per cent glycerol saline and held at 2° C Both reagents were diluted for use in saline, usually in a geometric series, using Grade A pipettes, burettes and volumetric flasks The cumulative errors of serial dilution were avoided as far as possible by adding " master " solutions to various volumes of saline in large vessels Thus, for a 2-fold series the volumes in ml respectively of toxin master-solution and saline were 3 + 3 , 2 + 4 , 1 + 7 , 1 + 15 , 1 + 31 , 1 + 63 , 1 + 127

Except when otherwise stated, laboratory stock albino guinea-pigs of 250 to 400 g weight were used , for any one test the weight range was usually within 80 g The animals were clipped, depilated by a barium sulphide paste 18 hours before the tests, and all animals with the slightest evidence of a depilation " burn " were rejected. A minimum of three animals was used for each titration Intradermal injections of 0 1 ml were made with needles of No 26 gauge

The intensity and diameter of areas of inflammation were measured after 24 hours, correct to the nearest half-millimetre The diameter of all round lesions was recorded, and the major and minor diameters (D and d) of the remaining slightly elliptical lesions reduced to a single measure, $\sqrt{Dd}$ The responses to series of intradermal doses of toxin were subjected, when necessary, to regression analysis (Fisher, 1947)

EXPERIMENTAL

The dose-response to intradermal diphtheria toxin

Current methods of titration usually depend on the determination of an endpoint, which is embodied in the conception of the minimal skin-reacting dose

In practice this appears to be the amount of toxin producing an inflammatory lesion between 8 and 15 mm in diameter For example, Jensen's (1933) " DRM " gave a lesion 8 to 10 mm in diameter Amounts of toxin producing lesions larger than 30 to 35 mm are impracticable because of the ultimate, perhaps fatal, general intoxication following the doses necessary to produce lesions of this size , and lesions smaller than 6 mm may be confused with the faint but definite area of inflammation, 3 to 4 5 mm in diameter, induced by injecting 0 1 ml of 0 85 per cent saline Between these two limits there is a practicable range of doses and, since estimates of toxicity depending on the responses to more than one dose are preferable to those depending on an end-point, the dose-response to diphtheria toxin in the skin was investigated within this range
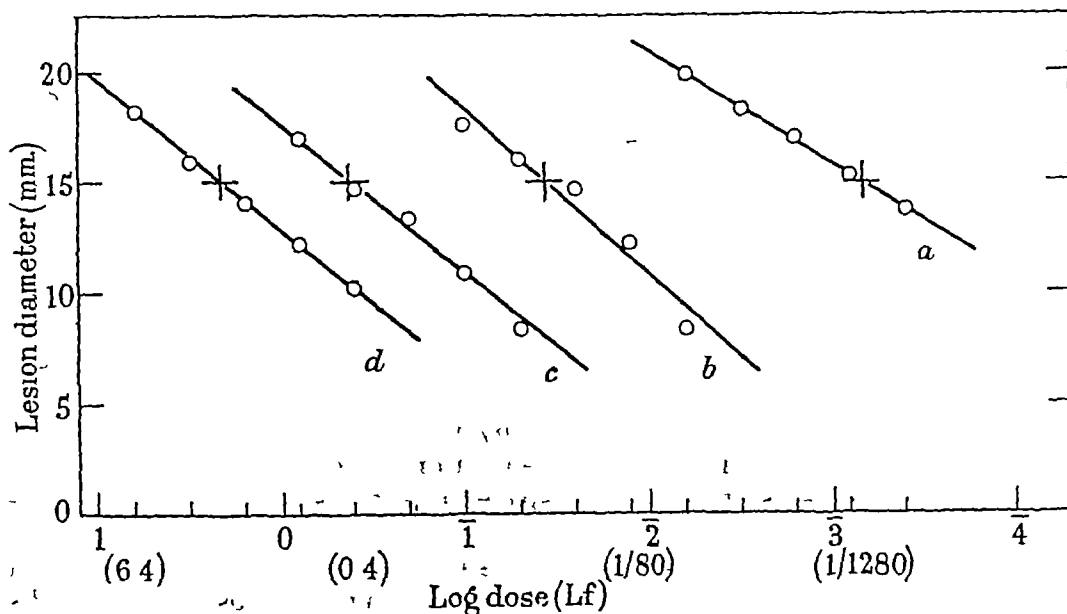

FIG 1 —Indirect neutralization of intradermal diphtheria toxin by antitoxin Linearity and parallelism of the dose response curves

(a) Antitoxin nil
(b)   ,,   60 u per kg guinea-pig body weight
(c)   ,,   600 u ,, ,,   ,,   ,,   ,,
(d)   ,,   6000 u ,, ,,   ,,   ,,   ,,

The dose-response lines were fitted from the means of six lesion diameters in three guinea-pigs

The curve obtained by plotting diameter (d) of the 24-hour inflammatory area against the logarithm of the dose (x) proved to be linear (Fig 1, 2a, 4) , that is, $d = b \log x$, where b is the slope of the regression line The slope was remarkably reproducible , for example, in 13 assays picked at random from a series made over 7 months, the slopes were − 6 75, − 7 59, − 5 97, − 5 94, − 7 03, − 5 21, − 5 91, − 6 78, − 5 81, − 7 59, − 6 57, − 6 54, − 5 23 The relative constancy of slope may depend on the age of the lesion, the batch of toxin and the animal used Jensen (1933) measured lesion-diameters in rabbits at 36 hours His Table 7 records titrations of a toxin in 15 rabbits , the diameters are given correct to the nearest 2 mm (i e 8, 10, 14, etc) By regression analysis of Jensen's figures, the dose-response proved to be linear in 12 of 15 rabbits, and in these animals the slope varied between 10 6 and 27 5

It was important to prove that this convenient linear dosage-response relation held for all parts of the guinea-pig skin used in the titrations For a given dose of toxin in 0 1 ml , the lesions in the ventral half of the body skin are bigger than

TABLE I —*The Analysis of Variance of the Diameter of the Toxin Lesions in the Skin of Six Guinea-pigs in Response to Four Doses of Diphtheria Toxin, Randomized in a Four-fold Latin Square*

Source of variation	DF	Mean square error	F	P
Between AP* rows	3	7 1485	4 71	0 001–0 01
,, T* rows	3	2 2596	1 49	>0 05
,, doses	3	347 5859	228 9	<0 001
Linearity	1	1,028 1380	677 2	<0 001
Departures	2	7 3099	4 81	0 01–0 05
Between guinea-pigs	5	1 9422	1 28	>0 05
Interactions	45	1 8861	1 24	>0 05
Residual	36	1 5182		
Total	95			

* See p 322

in the dorsal half Titrations were therefore confined to an area on the two sides of the spine, bounded laterally in the sitting animal by the horizontal lines midway between the spine and the anterior belly wall, anteriorly by the vertical line through the elbow-joint, and posteriorly by the vertical line through the knee-joint Within this approximately square area on the dorsal aspect of the animal it was possible to make sixteen equally spaced injections, 2 rows of 4 on each side of the spine

The linearity of the dose-response and its independence of the site of injection within the chosen area were established by titrating four doses of toxin distributed randomly among the 16 available sites in Latin squares Three tests were made, each in 6 to 8 animals Table I records the analysis of variance from one test, typical of the three Doses of 1/400, 1/1600, 1/6400 and 1/25,600 Lf were given to six guinea-pigs, so that each dose was tested 24 times From column F (the mean square errors in terms of the residual mean square error 1 5182) it is clear that the variation due to position in the injection area, indicated by the variations in the response in the four antero-posterior (AP) rows of lesions is small compared with the difference between doses, and that in the four transverse (T) rows it is negligible In two similar tests the variation between both T and AP rows was negligible For practical purposes the skin of this area may be considered homogeneous with respect to toxin In most subsequent tests, therefore, no formal randomization of doses among the available sites in all the animals in each test batch was attempted, though the pattern of injections was varied when possible In this example the variation between animals is small , in other tests it was larger, and sometimes significant , but in all the cases analysed each animal provided a substantially linear response and the skin was uniformly susceptible to the toxin

The value of F for linearity is satisfactorily significant, and for departures from linearity it is small It follows that, by the simple transformation of dose

into log dose, the information given by the mean response to all the doses injected can be combined to obtain single estimate of toxicity   For this purpose a regression line is fitted and log x determined for an arbitrarily selected diameter of lesion. Values between 12 5 and 15 mm. were chosen, being near the mid-value of lesion-diameters usually obtained in the titrations   They are rather larger than the diameters produced by the orthodox " minimal reacting dose," but for two reasons they are more reliable measures of toxicity , they summarize the response, not to one dose, but to a series of doses , and they do not depend on personal judgement as to what a standard minimal reacting dose is   In this connection it may be noted that the lesion-diameter of a single dose is not a very sensitive indicator of variation in toxin concentration   The average slope is about -7 , and since $d = -7 \log x$, the lesion diameter diminishes only by 2 1 mm even for a 50 per cent decrease in concentration

The dose-response to indirectly neutralized toxin

The dose-response to toxin in animals with circulating antitoxin is also linear, and the slope is parallel to that for toxin alone, even in animals receiving very strong antitoxin   Each fitted slope in Fig 1 summarizes six titrations, two per guinea-pig   Similar results were obtained in two repetitions of the test   The linearity and parallelism of the slopes are obvious , and on analysis the four regression lines were homogeneous, the deviation of each from the common regression line being insignificant   Taking a 15 mm lesion for the point of comparison, the relative toxicities are as follows   60 u antitoxin per kg diminishes toxicity 51 4-fold , 600 u , 11 4 × 51 4-fold , and 6000 u , 5 16 × 11 4 × 51 4 = 3,023-fold   The 5 16-fold decrease in toxicity for the 10-fold increase in antitoxin injected (600 to 6000 u) compared with the 11 4-fold decrease for the step 60 to 600 u suggests that with very strong intradermal toxin the constant proportionality between toxin and antitoxin, noted by Friedemann (1947) in the rabbit, does not hold (see p 326)

At the lower end of the curves, where lesion-diameters are <6–8 mm , there was sometimes a departure from linearity, the curves dipping more steeply to the 3–5 mm values   These values, covering the range of diameters of the mild inflammatory lesions produced by saline alone, must, for practical purposes, be considered as the base line of non-toxicity   Nevertheless, as in Fig 5, I, with low concentrations of circulating antitoxin, the curves meet this base line at points whose distance apart is equal to the log decrease in antitoxin concentration   That is to say, with concentrations of toxin up to 1000 times that giving a 12 5 mm lesion in the normal animal, the degree of indirect neutralization is proportional to the concentration of circulating antitoxin, whether the neutralization is measured by shift of the dose-reponse slope or, as Friedemann and his colleagues found, by shift of end-point   But with very strong toxin, simple proportionality does not hold, and antitoxin is less efficient

The dose-response to directly neutralized toxin

The picture is strikingly different for in vitro mixtures of toxin and antitoxin , Fig 2 is typical of the dose-responses to mixtures of a constant amount of antitoxin and graded amounts of toxin, held at 37° C for 1 hour before injection   They differ sharply from those in Fig 1 , the upper part is approximately parallel

to that of toxin alone, but for lesion-diameters less than 17 to 18 mm , the curves dip steeply to the base-line at 3 to 5 mm   In the similarly steep portions of the curve that sometimes occurred in indirect neutralization (Fig 5, I) the change of direction occurred only at lesion diameters of 6 to 8 mm , i e close to the lower end of the dose-response line

As in Fig 1 and 5, I, the ratio of these end-point values is equal to the ratio of antitoxin concentration used   From several titrations of this kind, the slope of this lower part of the curve was estimated as approximately − 35, a value
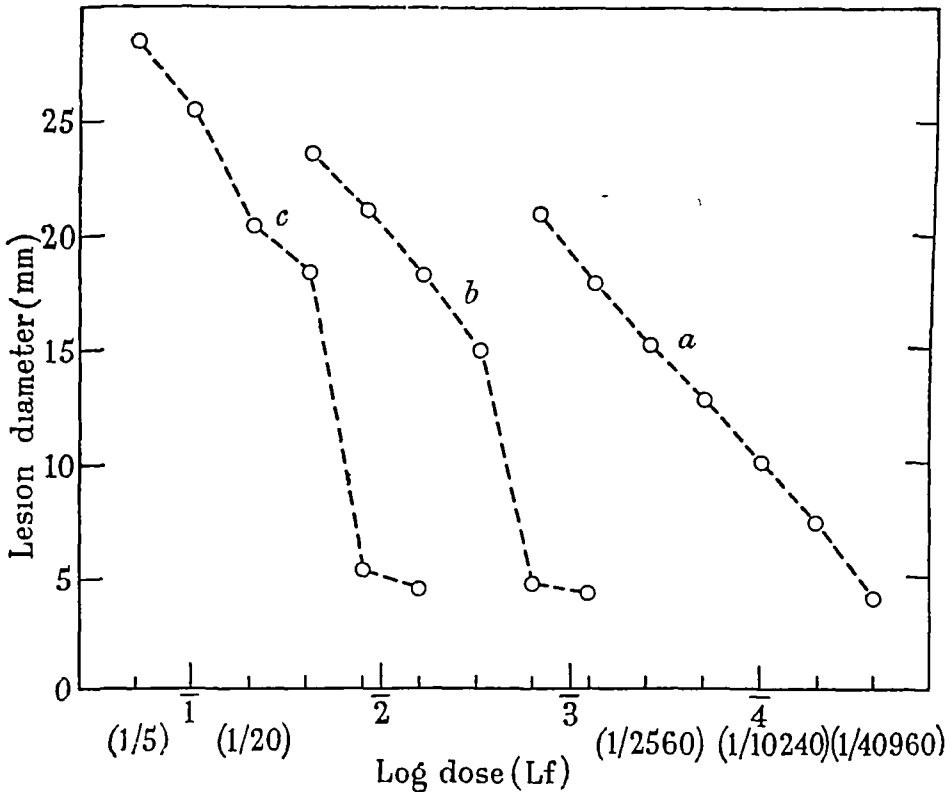

FIG 2 —Direct neutralization of intradermal diphtheria toxin by antitoxin   The non-linearity of the dose response curve, and absence of parallelism with the curve for toxin alone
(a)   Antitoxin nil
(b)   ,,   0 0025 u
(c)   ,,   0 02 u.

that differs significantly from the slope for toxin alone or for indirectly neutralized toxin, which ranged from − 5 to − 9   It is presumably the steepness of the slope in direct neutralization that permits accurate intradermal measurement of antitoxic potency by titration against a fixed amount of toxin, since an end-point can be estimated with fair precision

The dose-response to toxin indirectly neutralized by late-administered antitoxin

Returning to indirect neutralization, we find that variation in the period between injecting toxin and antitoxin has a notable effect which is readily measured in the same animal by injecting toxin at varying times before and after the intravenous injection of antitoxin   Comparison of the dose-response to

toxin indirectly neutralized by antitoxin given before and after toxin, established the following facts

With small doses of toxin, requiring relatively little circulating antitoxin, there is no significant difference between the curves for antitoxin given 6 hours, 2 hours, and 3 minutes (— 3) before the toxin, and 3 minutes after (+ 3) the toxin Antitoxin given even 60 minutes later (+ 60) has considerable neutralizing power (Fig 3, typical of several tests) By regression analysis, the difference between the — 3 and + 3 curves was not significant, and between — 3 and
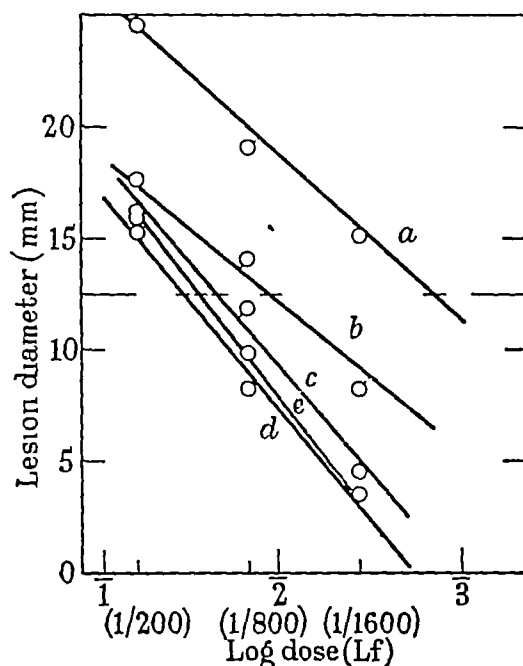

Fig 3 —Indirect neutralization of intradermal diphtheria toxin by 1 5 u antitoxin per kg Similarity of neutralizing potency of antitoxin injected three minutes before and after the toxin

    (a)   Toxin in animals without antitoxin
    (b)   „   given 60 minutes before antitoxin
    (c)   „   „   30   „   „   „
    (d)   „   „   3   „   „   „
    (e)   „   „   3   „   after   „
The dose response lines were fitted from mean lesion diameters in five guinea pigs

+ 30 only just so , and, taking a lesion-diameter of 12 5 mm for comparison, the potencies of the toxin in terms of its — 3 value, and the fiducial limits of error were

Antitoxin	Potency of toxin	Limits (P = 0 95)
None	12 28	7 41–21 27
60 min late	2 50	1 53–4 18
30 min late	1 37	0 83–2 25
3 min late	0 84	0 51–1 38

Thus, even when the antitoxin is given 60 minutes late, only 2 5/12 28 = one-fifth of the toxin is not neutralized

The constant proportionality of toxin and antitoxin, already noted in Fig 5, I, where the antitoxin was given at − 10 (i e 10 minutes before toxin), is disturbed when antitoxin is given late   In Fig 5, II, with antitoxin given at + 10, the ratios of toxicity with the different antitoxin levels, as far as can be estimated, are much less than the expected value of 4

In Friedemann and Zuger's (1939) Tables 3 and 4, the greatest effect of the late administration of antitoxin occurs with the strongest antitoxin   This is also evident in Fig 4, where with 1 u antitoxin per kg the neutralization curves at − 12 and + 60 differ little, whereas the curve for 9 u antitoxin per kg has
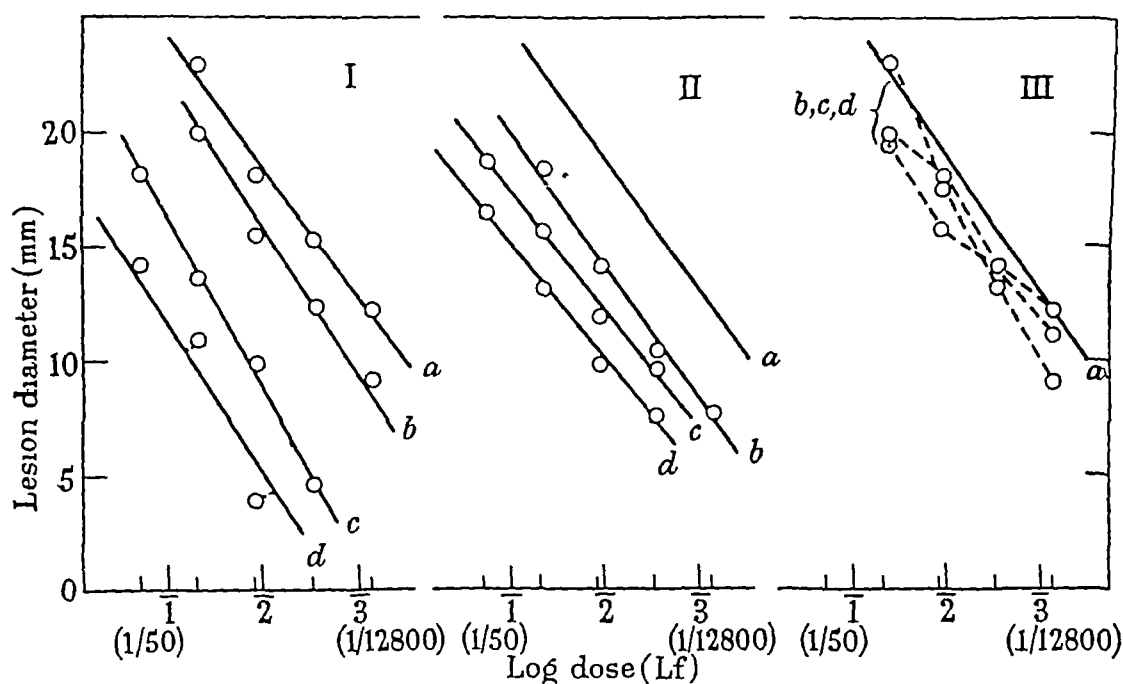

FIG 4 —Indirect neutralization of intradermal diphtheria toxin by antitoxin   Increasing loss of neutralizing potency with increasing lapse of time between giving toxin and antitoxin
         I   Toxin given   12 minutes after antitoxin
         II   ,,   ,,   60   ,,   before   ,,
         III   ,,   ,,   120   ,,   ,,   ,,
(a), (b), (c) and (d) respectively are the dose response lines from guinea pigs receiving 0, 1, 3 and 9 u antitoxin per kg

moved up considerably at + 60   The effect is presumably a consequence, not of the high concentration of antitoxin in the plasma, but of using the strong toxin, which in turn entails the use of the strong antitoxin   Even with pre-administered antitoxin, proportionality is disturbed when the toxin is very strong (Fig 1d and c), and this disturbing effect of strong toxin appears to be exaggerated when the antitoxin is injected late   Fig 4 also illustrates the fact that the indirect neutralizing power of antitoxin is small when the toxin lesions are two hours old

These results offer little ground for supposing that a sharp distinction can be made between indirect neutralization by antitoxin given before and after toxin At the critical point, the moment of injection of toxin, the change is gradual The effect, however, is not symmetrical about this point, for whereas the diffe-

rence between antitoxin given 3 and 120 minutes early is negligible (see also Friedemann and Zuger, 1939), that between 3 and 120 minutes late is striking

The Implication of the Dose-response to Diphtheria Toxin

The results so far reported largely confirm those of Friedemann and his colleagues   There are, nevertheless, certain features of the dose-response curves for directly and indirectly neutralized toxin which suggest several objections to Friedemann's hypothesis
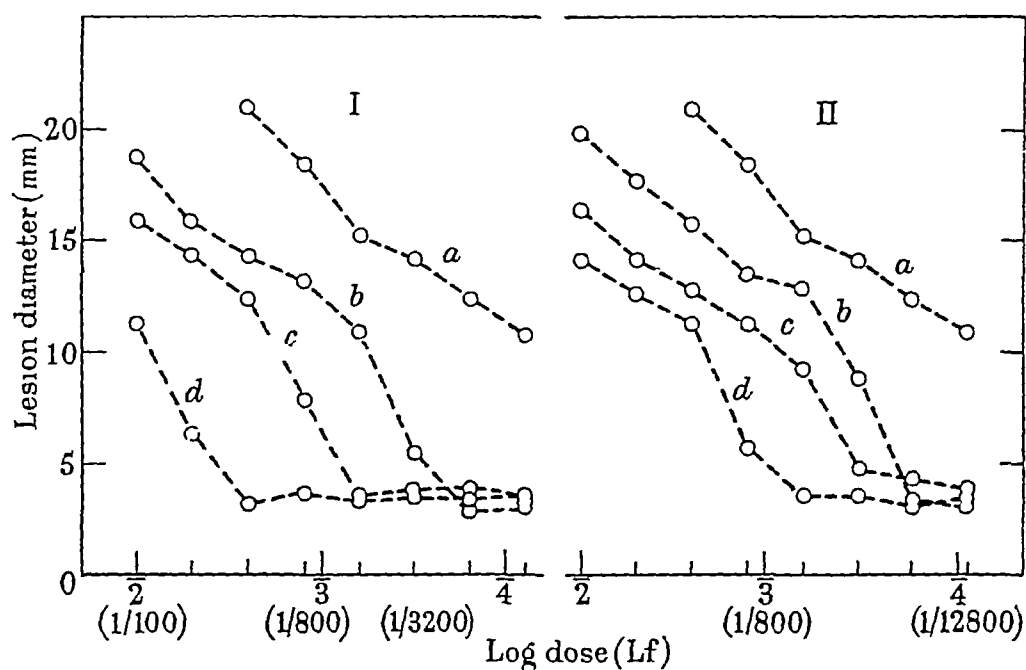

Fig 5 —Indirect neutralization of intradermal diphtheria toxin by antitoxin   The effect of late administration of antitoxin on the constant proportionality of skin toxicity and blood concentration of antitoxin.

I   Toxin given 10 minutes after antitoxin
II   „   „   10 „   before   „
(a), (b), (c) and (d) are respectively the dose response curves from guinea pigs receiving 0, 1 5, 6 and 24 u. antitoxin per kg

Direct neutralization

The characteristic change of slope in the dose-response to mixtures of toxin and antitoxin may be simply explained   In Fig 2b the lesion from 1/640 Lf is diminished to the size of that of 1/40960 Lf (i e a 64-fold drop) by 0 0025 units antitoxin, which therefore neutralizes 63 of these minimal doses (m d) of 1/40960 Lf   Since the antitoxin in these toxic mixtures may be assumed to be fully saturated with toxin, we may set down a series of doses of toxin in terms of this m d , increasing 2-fold , and if from each figure we subtract 63, equivalent to the neutralizing power of the antitoxin added, we have as follows

Toxin (m d)	Antitoxin added	Free toxin	Ratio dose of free toxin to preceding dose
16	63	0	
34	63	0	
64	63	1	∞
128	63	65	. 65 00
216	63	193	2 97
512	63	449	2 28
1024	63	961	2 18
2048	63	1985	2 07

Instead of a constant doubling of dose of free toxin, the effective doses (Column 3) increase rapidly at first, and then, as the ratios in Column 4 show, approximate more and more to the 2-fold increase   The response to such a series would correspond closely to that found (Fig 2b), and, moreover, with stronger antitoxin, the length of the steeper part of the hypothetical dose-response curve would be increased, as happens in Fig 2c

The large difference between the slopes of the dose-response to toxin neutralized directly and indirectly (about − 35 and − 7) means that Friedemann's ratio λi Ad, will vary with the degree of inflammation chosen as end-point in the toxin titrations   In Fig 2a the slope for toxin alone represents the slope for indirectly neutralized toxin, since (Fig 1) the one is parallel to the other , and a comparison of this slope with those for direct neutralization clearly shows how the non-parallelism will affect estimates of antitoxic efficiency at different lesion-diameters   Even if consistent results were obtained with a well-defined end-point due to weak toxin, the ratio would not be a valid basis for deducing a blood tissue ratio of antitoxin in lesions due to stronger toxin

Indirect neutralization

Friedemann s hypothesis postulates the establishment, very soon after its injection, of a protective layer of antitoxin of " molecular dimensions " over the susceptible tissues of the skin   The tissue antitoxin must in this case be uniformly distributed throughout a uniformly reacting tissue like the guinea-pig's skin   In contrast, the distribution of intradermal toxin, injected from a single point in the skin is unlikely to be uniform , and it will be profitable to consider what it is, and how it will affect indirect neutralization   The distribution of toxin will depend on the one hand on the absorbing power of the tissues, and, on the other hand the outward flow of toxin solution from the needle-point first under the pressure of injection, then under the pressure of the tissues distended with liquid by the force of injection and, when these mechanical forces are spent, by diffusion and other molecular forces   On immediate injection, 0 1 ml of fluid regularly raises a bleb 10 0 to 11 5 mm in diameter   One hour later a flat 15 to 18 mm bleb is recognizable under oblique illumination of the skin surface , and after a few hours the fluid is mechanically spread over an area about 20 mm in diameter   It is questionable how far diffusion, etc , is operative, but lesion-diameters of up to 30 mm accord with the linear relationship described above, though they are presumably due to toxin spread by means other than the injec-

tion pressure   But in lesions up to 20 mm in diameter, the distribution of effective toxin may be assumed to be the resultant of adsorption of toxin and mechanical dispersion of the toxin solution alone , and for this part of the curve, the molecular dispersing forces may be neglected

It is experimentally obvious that the distribution of toxin is not uniform, because, with increasing dose of toxin, the inflammation becomes not only more intense, but disproportionately more intense in the centre of the lesion—an effect commonly seen in lesions following the intradermal injection of many toxic substances   The concentration, then, may be assumed to be highest in the centre, and in each lesion there must be finally established, from the centre outwards, a concentration gradient of toxin ending with the minimal intoxicating concentration at the periphery   There are no data for determining the shape of this gradient   It may resemble those in Fig 6a, b or c, or it may be a more or less exaggerated version of one of them   It is convenient to assume a gradient with a feature common to all three, namely, a straight line, and later to consider whether conclusions derived from this assumption are valid for other hypothetical gradients

Calculation of hypothetical linear concentration gradients—Fig 6d and e are median cross sections of lesions in which the concentration gradient is linear, the abscissa represents the diameter, and the ordinate the concentration of toxin The total dose of toxin is therefore represented by a cone whose base is the area of the lesion, whose height is expressed in arbitrary units of toxin and whose volume is $\pi/3$ (area $\times$ height)   Part of the toxin injected is ineffective because of the basic natural immunity of the skin tissue   The distribution of toxin in the maximum ineffective dose is represented in Fig 6d by the shaded triangle whose base is $2z$, where z is the radius of the circle over which the toxin is distributed, and whose height is Ci, the maximum concentration of *ineffective* toxin at the centre of the injection site , Ci is therefore a measure of the basic natural immunity of the skin, and is uniform throughout the skin area used in the titration of toxin (p 322)   When the central concentration of toxin is $>Ci$ (Fig 6e) measurable inflammation occurs , r is the radius of the lesion, and Ce is the central concentration of *effective* toxin   The maximum ineffective dose (m i d) of toxin is determinable by extrapolation of the dose-response line to zero lesion-diameter, and is a convenient unit measure of toxin   From Fig 6d, 1 m i d $= z^2\,Ci\ \ \pi/3$ Expressing the dose of toxin as x m i d , when $x>1$ we have from Fig 6e

$$x = z^2\,(Ce + Ci)\ \ \pi/3$$

Since $\pi/3$ is a constant and nearly unity (1 047) the equation may be written

$$x = z^2\,(Ce + Ci) \tag{1}$$

The effective part (Te) of the dose x is clearly

$$Te = r^2 Ce$$

But from the figure

$$z = r(Ce + Ci)/Ce$$

Substituting in (1) we have

$$x/r^2 = (Ce + Ci)^3/Ce^2 \tag{2}$$

In this equation x and r are determinable, z is eliminated, and Ci is by definition a constant

As a numerical example we may take a dose-response line determined early in this work in which a two-fold decrease in the dose x decreased the radius by 1 5 mm By extrapolation the m i d was 1/512,000 Lf In the expression for the dose response line $d = b \log x = 2r$, b is therefore $- 10$ and $r = - 5 \log x$ Since the slope b is negative only because the graphs record increasing dilution of toxin from left to right, we may omit the minus sign and write $r = 5 \log x$ Applying this to (2) we find by differentiation that the minimum value of x/r^2 is 0 391 and $Cc = 0 058$ m i d One m i d of toxin is therefore $0 058z^2$, and z, the radius of spread of ineffective toxin, is 4 15 mm That is, 1/512,000 Lf of toxin preads over an area 8 3 mm in diameter

With regard to effective doses of toxin, there is no simple solution of equation (2) to get Cc in terms of x/r^2 and Ct The values in Table II were obtained empirically When, for example, 1000 m i d are injected, the lesion diameter is 30 mm and 96 per cent of the dose is toxic, with a dose of 4 m i d only 14 7 per cent is toxic, and produces a lesion 6 mm in diameter The toxin gradients for this series of doses is illustrated in Fig 6f The most striking feature is the highly disproportionate increase in Ce as the dose increases, if the toxin is to be accommodated in lesions of the diameters observed experimentally Ce in lesions 15 and 30 mm in diameter is 5 5 and 65 6 times that in a lesion 6 mm in diameter

TABLE II —*The Relation Between Lesion-Diameter and the Distribution of Toxin After Intradermal Injection (see Fig 6f)*

Radius of lesion (mm) r	Total dose of toxin (m i d) x	Central concentration of effective toxin (m i d) $x/r^2 = Cc$	Amount of effective toxin (m i d) $r^2Cc = Tc$	Percentage of x that is effective $100(x - Tc)/x$
1	1 58	0 016	0 016	1 1
3	3 98	0 065	0 585	14 7
5	10 00	0 150	3 750	37 5
7 5	31 62	0 358	20 137	63 7
10	100	0 814	81 400	81 4
12 5	316 2	1 820	284 375	89 9
15	1000 0	4 268	960 300	96 0

For explanation of symbols, see p 329

The importance of this numerical demonstration, which serves mainly to emphasize the experimental commonplace of disproportionately increasing severity in the centre of skin lesion with increasing dose of toxin, lies in testing the validity of Friedemann's hypothesis of an immediately established, definitive concentration of antitoxin in the tissues Translated into the terms used above, the hypothesis postulates that immediately after intravenous injection of antitoxin, Ct is raised uniformly throughout the skin and that no subsequent increase in Ct is effective Thus in the experiment summarized in Fig 1, an intravenous concentration of 60u antitoxin per kg decreased the potency of the toxin 51 4-fold on Friedemann's hypothesis Ct was increased 51 4-fold But it is clear also from Fig 1 that the slope for toxin alone is almost parallel to that for toxin

indirectly neutralized by 60 u per kg , the two slopes were respectively — 5 23 and — 7 45, and did not differ significantly from one another   These two statements, however, are not compatible with the relation of b and Ci in equation (2)   Since $r = b/2$ (log x), we may rewrite the equation

$$4x/b^2 \log^2 x = (Ce + Ci)^3/Ce^2.$$

Both these functions have the same minimum , and since the minimum of $4x/b^2 \log^2 x$ is a multiple of $1/b^2$, and the minimum of $(Ce + Ci)^3/Ce^2$ is a multiple of Ci, $Ci \times b^2$ is a constant, and

$$b^2 \, \sigma 1/Ci$$

That is, as Ci increases, b, the slope of the dose-response line, decreases   Given — 5 23 as the slope for toxin alone, the slope for indirectly neutralized toxin should, on Friedemann's hypothesis, be $— 5\ 23/\sqrt{51\ 4} = — 0\ 73$, a sevenfold diminution   Now, the estimates of the two slopes in Fig 1 are subject to error, but the regression analysis would certainly have detected as heterogeneous any slope differing by a factor even of two, from that of the dose-response to toxin alone

Non-linear concentration gradients   The values for $Ci + Ce$ derived from equation (2), for the concentration of toxin at the centre of the lesion provide a means of testing whether toxin is absorbed to the tissues according to Freundlich's absorption isotherm   The constant absorbing surface is represented by a narrow cylinder of tissue at the centre of the lesion, and the amount absorbed is $(Ci + Ce)$ The concentration of toxin added to this system is x in the notation used above   The isotherm is

$$(Ce + Ce) = ax^n$$
$$\text{or } \log(Ci + Ce) = \log a + n \log x$$

where a is a constant and $n < 1$, and the data may be considered to fit if the relation between $\log(Ci + Ce)$ and $\log x$ is linear   Table III lists the relevant data from Table II   The plot of values in Columns 3 and 5 lie close to a straight line   The approximate value of n, however, is > 1, being 1 15 , implying that the greater the concentration of toxin, the greater the proportion of toxin absorbed   Consequently, if the isotherm applies, the acutely peaked distributions in Fig $6f$ are highly unlikely   To meet this difficulty we may assume that at the centre of the lesion a substantial core of tissue contains the maximum concentration of toxin   That is, the distribution is in the form of a truncated triangle, and approximates more to Fig $6a$ or b

TABLE III —*The Absorption of Toxin at the Centre of Skin-lesions in Terms of Dose Injected   Freundlich's Absorption Isotherm*

r (mm)	$Ce + Ci$ (m i d)	Log $(Ce + Ci)$	x (m i d)	Log x
6	0 123	$\bar{2}$ 8129	3 9S	0 6
10	0 20S	$\bar{1}$ 1761	10 0	1 0
15	0 416	$\bar{1}$ 5539	31 6	1 5
20	0 S72	$\bar{1}$ 9106	100 0	2 0
25	1 S78	0 2601	316 0	2 5

23

Nevertheless, with regard to all the hypothetical concentration gradients other than the linear, it will be clear from the values of Ce in the table that a greatly disproportionate elongation of the distribution curve of toxin in the
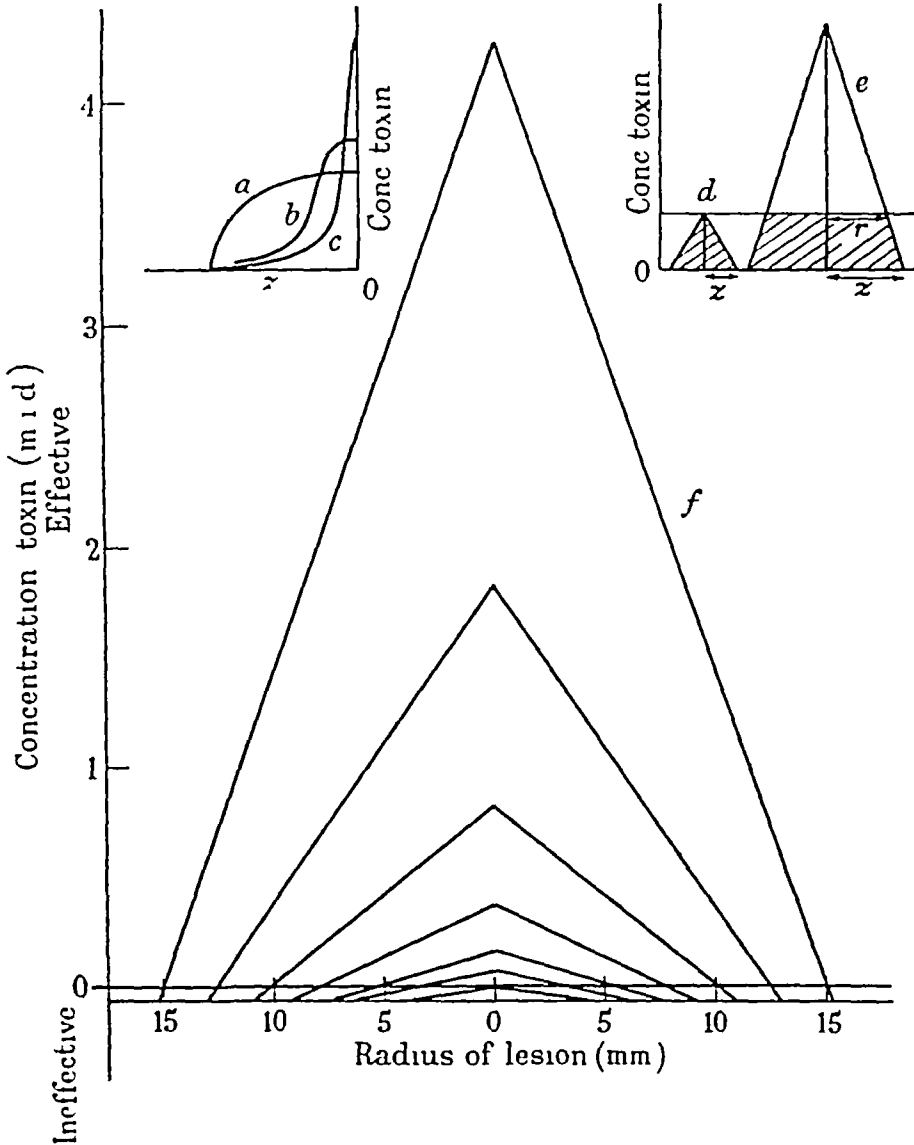

Fig 6 —Diagrams of the distribution of diphtheria toxin in median cross sections of skin-lesions after intradermal injection

(a) (b) and (c)   Possible types of the gradient of toxin concentration from the centre of the lesion

(d) and (e)   Distribution of a maximum ineffective dose (m i d), and of an effective dose of toxin with a linear concentration gradient

(f) Hypothetical distribution of graded doses of toxin, with linear concentration gradients, in guinea-pig skin lesions, for a dose response where $r = 5 \log x$ (Table II)

lesion will occur as the dose increases, whatever the shape of the distribution, and on Friedemann s hypothesis, therefore, that a decrease in slope is likely with increased Ci It might be possible to devise a concentration gradient, different from the simplified linear gradient from which equation (2) was derived,

such that the slope of the dose-response would remain constant with increasing C_i But two hypotheses are now required to explain the observed constancy of slope Firstly, that C_i is uniformly raised to a definitive level soon after the injection of antitoxin—a consequence of the properties of antibody globulin and capillary endothelium , and, secondly, that the distribution of a quite different substance—diphtheria toxin—in skin tissue is precisely adjusted to compensate for any rise in C_i from that of normal tissues to at least (Fig 5) 3000 times this value It is more economical of hypotheses to assume that the degree of indirect neutralization is determined primarily by the distribution of toxin, without any assumption about the establishment of a definitive concentration of antitoxin in normal tissues

Factors affecting indirect neutralization

The simplest assumption is that the degree of indirect neutralization, i e the rate at which antitoxin passes through the capillary walls, is a function of the concentration of toxin in the tissue , in other words, that toxin stimulates the passage of antitoxin Toxins like those of *Cl welchii*, *Cl septicum*, or the cobra rapidly increase the capillary permeability in the skin of the guinea-pig In animals with pontamine sky blue 6 X (75 mg per kg body weight) in the circulation, these toxins in 5 to 30 minutes induced intense staining of the lesion by exuded dye-stained plasma, the speed and area of exudation being greater with higher concentrations of toxin No such exudation occurred in diphtheria toxin lesions up to 7 hours old In 8- to 9-hour lesions there was slight exudation of coloured plasma in irregular areas round the periphery, and 20-hour lesions were readily permeable to circulating dye Gross capillary trauma in the first six hours may therefore be excluded

The trauma of injection The injection of 0 1 ml of 0 85 per cent saline, or of Ringer's or Locke's solution, did some damage, producing a faint blue stain 3 to 4 mm in diameter when the fluid was injected slowly in 2 to 3 seconds, and up to 5 mm when the fluid was injected as rapidly as possible to produce maximum trauma Since antitoxin might exude into the tissues by reason of the trauma, and affect the whole lesion, the magnitude of this traumatic leak was estimated

In the first place it takes place mainly in the first hour after injection, because when pontamine blue was injected into animals with saline lesions one hour old, the blueing was about 20 per cent of that in lesions made in animals with already circulating dye, showing that after 1 hour the tissues had largely recovered from the injection-trauma

Secondly, the volume of plasma leaking through is small The mean area and intensity of blueing after 1 hour was estimated in several lesions made by injecting 0 1 ml saline into two guinea-pigs with circulating pontamine blue Three ml samples of blood from both animals were then defibrinated, mixed and centrifuged, and the dyed serum diluted serially The undiluted serum was injected intradermally into fresh animals in 0 01, 0 015, 0 02 and 0 03 ml volumes from a microsyringe and the mean diameter of the blued areas measured to provide a standard volume-area relation At the same time 0 1 ml of the serum dilutions were injected to provide standards for intensity By direct matching of these lesions with the blue saline lesions, the volume of circulating

plasma required to produce the observed degree and area of blueing by exudation or leak could be estimated , in the two animals it proved to be 0 0013 and 0 0025 ml respectively These figures are reliable only as indications of the *order* of size of the plasma leakage induced by saline

Thirdly, antitoxin in volumes similar to that of the saline leak does not affect toxin lesions Guinea-pigs were injected with enough toxin to produce lesions 18 mm in diameter, and immediately afterwards, 0 005 ml volumes of anti-toxin solution (representing twice the volume of the estimated saline leak) were injected with a microsyringe into the centre of the blebs Antitoxin in con-centrations up to 2000 u per ml , which is far in excess of the plasma concentration
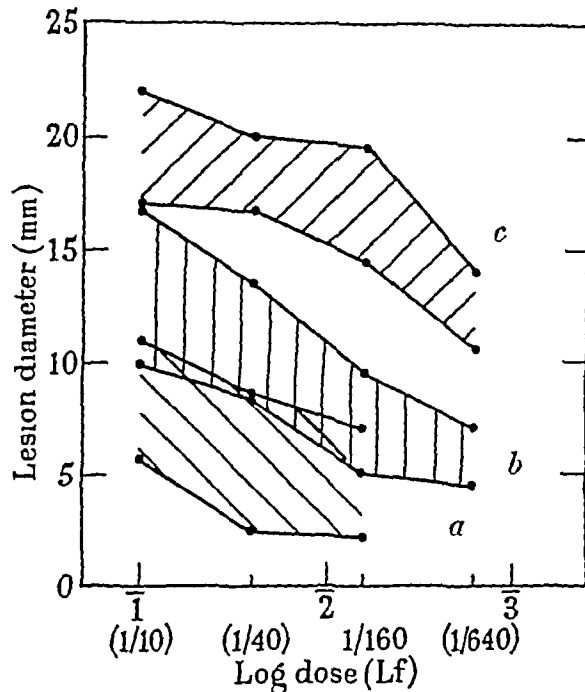

Fig 7 —The " transient zone " phenomenon following the intradermal injection of diphtheria toxin into guinea pigs with circulating pontamine blue The heights of the shaded areas represent twice the width of the zone of transient grey-blue coloration in lesions 2 hours (lower band), 3 hours (middle band) and 4½ hours old (upper band)

in indirect neutralization, failed to diminish the size of the lesion significantly This result incidentally suggests that diffusion of antitoxin from the centre of an injection bleb is exceedingly slow, for the amount injected was in some cases sufficient to neutralize several thousand times the amount of toxin present

For these reasons, the capillary permeability induced by the unavoidable trauma of injection is a negligible factor in indirect neutralization by antitoxin

The " transient-zone " phenomenon Certain changes, however, occur in the early stages of toxin lesions in animals with circulating dye As the lesion ages, a faint but definite grey-blue colour, more intense and darker than that of the surrounding skin, develops at the centre The area of this coloration expands, and at the same time the colour in the centre changes to a lighter blueish-pink, leaving a zone of grey-blue which expands till it reaches the edge of the lesion and then disappears Fig 7 epitomizes the essentials of this phenomenon of the

expanding, transient zone   The measures were made in guinea-pigs given 4 different intradermal doses of toxin at four different times, so that one hour after the intravenous injection of dye, when the readings were taken, the four titration series were respectively 1, 2, 3 and 4½ hours old   The grey-blue zone appears first after 2½ hours, in the centre of the lesion (where toxin is most concentrated), it is wider and appears earlier with the large doses of toxin, and reaches its maximum size in about 5 hours, and then gradually disappears   The phenomenon cannot be elicited easily in all guinea-pigs   Some show it in all lesions of the right age and others erratically, only in a few lesions, but in all the animals tested there was some sign of it, and it may therefore be taken as a characteristic feature of the reaction of guinea-pig skin to diphtheria toxin   It is not due to an impurity in the toxin because it did not occur with toxin mixed with a just-neutralizing dose of antitoxin   Nor is it likely to be a subliminal effect of any substance in the toxin which produces gross capillary permeability, because the toxin was used at dilutions of 1/640, and induced gross permeability only when 1/5 (containing 15 6 Lf per ml) was injected   The grey-blue colour was diminished by pressure, and appeared to be due to an intense transient vascular dilatation lasting about 1 hour in any one part of the lesion   The phenomenon is not the same as the "traumatic reaction" described by Jensen (1933) in rabbits, which is maximum in 6 hours with a diameter of only 4 mm, this also was observed in the guinea-pig lesions   It must be considered in the light of vascular physiology   Between the permeability of capillaries in normal skeletal tissue, permitting a slow passage of serum protein, and the gross permeability induced, for example, by histamine, peptone, certain bacterial toxins, and by acute inflammation (Field, Drinker and White, 1932), there may be a range of permeabilities characteristic of different physiological and pathological states   Whether in the transient zone there is any increase in the transendothelial passage of antibody can be decided only by direct demonstration of increased globulin exudation in these circumstances   Nevertheless, the toxin lesion obviously exhibits a substantial vascular abnormality in its early stages, which may be a factor in indirect neutralization

The rate of fixation of toxin in skin tissue

Though it is tempting to regard the gradually increasing inefficacy of late-administered intravenous antitoxin as a direct measure of the rate of fixation of toxin (Glenny and Hopkins, 1925), our ignorance of changes in capillary permeability in a progressive inflammatory lesion does not warrant it   For this reason, Wright and Clark's (1944) results are important and instructive   They injected antitoxin into toxin lesions already established in the human skin, and found that lesions 8 to 12 hours old were modifiable in some degree by the antibody   It is possible to elaborate his findings in the more accessible guinea-pig   The 0 1 ml volumes of toxin were injected through a fine needle coated on the outside with a dye and inserted in a determined direction   Through the needle-hole thus stained with dye it was possible to "superinject" antitoxin, using a slightly wider needle to avoid back leak along the old needle track and thus to ensure a distribution of both toxin and antitoxin solutions from the same point in the skin   The technique has certain drawbacks   Firstly, because the toxin may have spread beyond the confines of the injection-bleb by the time antitoxin is superinjected, a larger volume of antitoxin solution (0 2 ml) is

required if the antibody is to reach the limits of intoxicated tissue , and this volume of fluid, apart from its antitoxic effect, might drive unfixed toxin further from the point of injection, and create a potentially bigger lesion In these circumstances a superinjected lesion at 24 hours, of the same size as the control lesion, might be evidence of a considerable neutralization, though apparently indicating none To overcome this difficulty controls superinjected with saline were included in all tests

Secondly, a lesion superinjected immediately after toxin is necessary as a reference point In practice, the immediacy could not be approached nearer than 2 seconds, and 5 seconds in a series of injection, if a constant time interval were to be maintained Controls were also included of injections of 0 3 ml of the appropriate toxin-antitoxin mixture made *in vitro*, and of 0 1 ml toxin superinjected into blebs made by 0 2 ml antitoxin , but the conditions differed so much from the test lesions that only gross differences in results were significant

Fig 8, I, records the results of one test with a dose of toxin yielding a mean lesion-diameter of 19 3 mm Superinjected with saline, the lesion-diameter was 25 8 mm , the saline effect slowly declined as the superinjection was delayed, and was small after a 3 hours delay This phenomenon is itself evidence against an immediate fixation of toxin, but only on the assumption that the superinjected saline was dispersing the original diphtheria toxin, and not a secondary inflaming agent produced by the action of toxin on the tissues However, the neutralization by antitoxin superinjected at the corresponding times (lower curve) proves that the large " saline " lesion-diameter (S) is due to dispersion of the toxin itself, the "antitoxin" lesion diameter (A) is 13 6 mm less than S, and 7 1 mm less than the " toxin control " diameter

Though both upper and lower curves indicate a delayed fixation of toxin, they do not reflect the rate of fixation Neutralization by superinjected antitoxin is direct, and at first sight the difference, S — A, should lie on a dose-response curve similar to that in lower part of *b* and *c* in Fig 2 That is, on a slope of about — 35, when the ratio (R) of potencies of neutralized and un-neutralized toxin would be antilog $[(S — A)/35]$ The S lesion, however, is not a valid control for this purpose If the immediately-superinjected 0 2 ml saline merely diluted the 0 1 ml toxin solution already present, the resulting lesion should resemble that produced by the same amount of toxin injected in a volume of 0 3 ml But a change from 0 1 to 0 3 ml in the volume in which a given amount of toxin is injected does not alter the size of the lesion For example, eight guinea-pigs were injected with four concentrations of toxin (1/400, 1/800, 1/1600 and 1/3200 Lf/ml) each in volumes of 0 05, 0 1, 0 2 and 0 4 ml The injection sites were partially randomized among the animals For each combination of toxin concentration and injection-volume, the mean lesion-diameters in mm were

Lf/ml	Volume injected (ml)			
	0 4	0 2	0 1	0 05
1/400	26 9	20 4	16 5	12 5
1/800	21 3	16 1	11 5	10 5
1/1600	16 8	13 0	9 4	7 0
1/3200	11 5	10 2	7 9	4 6

Reading diagonally upwards from left to right, it will be seen that the same *amount* of toxin produces approximately the same lesion-diameter   Thus 0 4 ml of 1/1600, 0 2 ml of 1/800 and 0 1 ml of 1/400 give lesions 16 8, 16 1 and 16 5 mm in diameter   The increase in size by superinjected saline is therefore due to a mechanical displacement of toxin, which must to some extent be pushed outwards by the saline, piston-wise, along the narrow intercellular channels forced open by the injection   The concentration gradient of toxin that characterizes a single injection of toxin, and consequently the dose-response, is therefore disturbed by superinjection, so that the mean slope of — 35 is not valid.
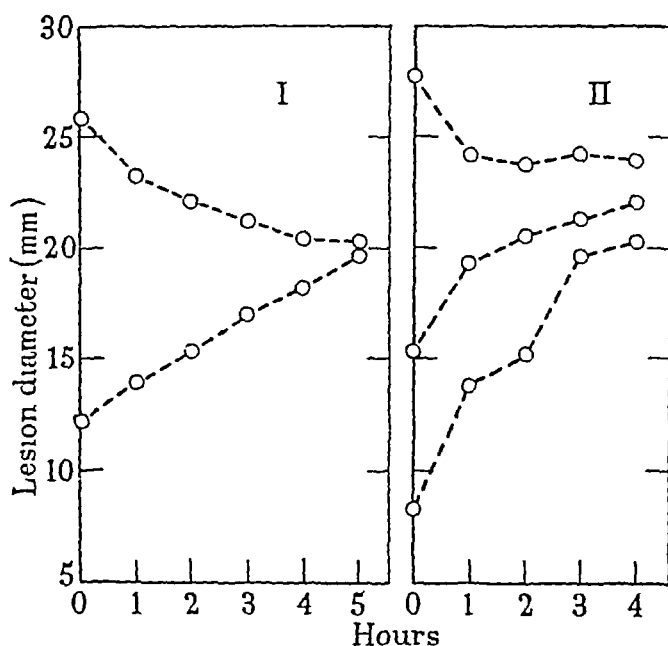

Fig 8 —The effect in the guinea pig of superinjecting saline (uppermost curve) and antitoxin into diphtheria toxin skin-lesions of various ages
  I. Toxin Lf 1/1000, antitoxin 0 002 u
  II. ,, Lf 1/ 250, ,, 0 002 u (middle curve) and 0 128 u (lower curve)
 Each curve is the mean from three guinea pigs, each with two sets of lesions

But saline has little effect in lesions 2–3 hours old, so that in these the initial concentration gradient must still obtain   The value of (S' — A) is therefore a better measure of decrease in toxicity, where S' is the mean value of S in lesions superinjected with saline after 3 or more hours   Antilog [(S' — A)/35] is then a better measure of R, the ratio of potencies of neutralized and un-neutralized toxin   If at each reading (0, 1, 2   .   etc hours) the potency of toxin in the S' lesion is taken as unity, that in the A lesion is 1/R, and 1 — 1/R is the proportion of toxin neutralized   Putting the value of 1 — 1/R at 0 hours = a, when neutralization by superinjected antitoxin is maximum, the percentage neutralization after longer periods in terms of this maximum is 100(1 — 1/R)/a   These values from the experiment illustrated in Fig 8, I, are listed in Table IV.

In another experiment (Fig. 8, II) it was clear that, compared with weak antitoxin (0 02 u /ml), antitoxin 64 times as strong (1 28 u /ml) could affect toxin even in lesions 5 and 6 hours old   But the general effect of the two con-

TABLE IV —*The Calculation of Percentage Neutralization by Antitoxin Super-injected into Intradermal Toxin Lesions of Various Ages (see Fig 10)*

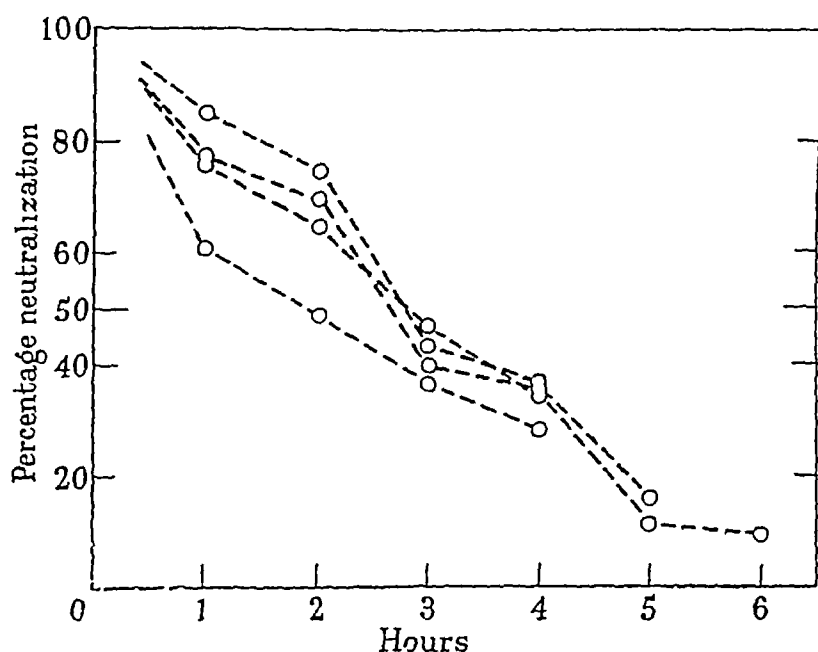| Superinjection after (hours)	(S' — A) (mm)	Ratio toxicity A and S' lesions R = antilog (S' — A)/35	Proportion toxin neutralized $p = 1 - 1/R$	Neutralization as percentage of maximum possible $100p/0\ 429$
0	8 47	1 75	0 429	100 0
1	6 89	1 57	0 363	85 0
2	5 64	1 45	0 320	74 5
3	3 72	1 28	0 185	43 2
4	2 49	1 18	0 152	35 5
5	1 14	1 08	0 068	15 8

For explanation of symbols, see p 337

FIG 9 —The rate of fixation of intradermally injected diphtheria toxin, as indicated by the percentage decrease in the neutralizing potency of superinjected antitoxin with increasing age of lesion

centrations of antitoxin is the same, as Fig 9 shows, where the percentage neutralization from the two experiments summarized in Fig 8, I and II, and from a third experiment with 0 02 u /ml of antitoxin, are plotted In all, there is in the first few hours a similar proportional diminution of neutralizing power of antitoxin with the age of the lesion at the time of superinjection, and, by implication, a roughly constant rate of fixation of toxin to the tissues The conclusions of previous investigators (e g Schick, Kassowitz and Buracci, 1916 , Glenny and Hopkins, 1925 , d'Antona, 1937 , Petherick and Singer, 1944 , Wright and Clark, 1944) about the slow rate of fixation in the skin are thus confirmed The rate in other, more susceptible, tissues is largely unknown, though the

experiments of Doenitz (1899) on the rapidly developing inefficacy of intravenous antitoxin after intravenous toxin, suggest that the skin may be exceptionally slow in this respect

The competition for toxin between tissue and antitoxin

The curves in Fig 8 demonstrate that whereas the concentration gradient resulting from a primary intradermal injection can be disturbed by saline superinjected up to 3 hours later, it is susceptible to modification by antitoxin for 2 to 3 hours longer   That is, toxin that cannot be shifted mechanically can be neutralized *in situ* by antitoxin, which presumably combines with already " fixed " toxin   Three stages in fixation in the skin are distinguishable

(1) Simple absorption of toxin to the tissues ; it takes place immediately on injection, and determines the concentration gradient of toxin in the skin, and therefore the nature of the dose-response revealed by inflammatory lesions 24 hours later

(2) Fixation of absorbed toxin so that it cannot be reversed by a limited fowl of saline ; it is complete within 2 to 3 hours of injection

(3) Fixation of absorbed toxin which is so firm that it cannot be reversed by antitoxin ; it is complete within 5 to 6 hours of injection   This degree of fixation is most important in antitoxin therapy

Between Stages (2) and (3) there is clearly competition for the toxin between tissues and antitoxin   Do these three stages occur in the indirectly neutralized skin lesion, and if so, is there competition in Stage (1) ?

When intradermal toxin is completely neutralized, it is theoretically possible that the injected toxin meets sufficient tissue antitoxin to neutralize it, and that antitoxin and tissues do not compete for toxin   But it is equally possible that some, and perhaps a large part, of the toxin is neutralized by antitoxin passing through the capillary endothelium after the injection of toxin ; and in this case there would necessarily be competition between tissues and antitoxin

In an animal with already circulating antitoxin that receives intradermal toxin, the removal of circulating antitoxin a few moments later should not affect the neutralization of the toxin if Friedemann's hypothesis is true   Any increase of lesion-diameter, compared with a control animal left with its circulating antitoxin, would suggest that neutralization continued after the injection of toxin   It may be objected that increase in lesion-diameter would in such circumstances not necessarily disprove Friedemann's hypothesis, since the antitoxin equilibrium is disturbed, so that the newly-formed toxin-antitoxin complex in the tissues dissociates, releasing toxin   But in this case it would clearly be necessary to postulate competition between tissues and antitoxin, and Friedemann postulates that there is none

The *rapid* removal of blood from a passively immunized guinea-pig and its substitution by antitoxin-free blood is technically difficult   The recent advances in methods of purifying diphtheria toxoid provide a means of avoiding this difficulty, namely, neutralization of the circulating antitoxin with concentrated, innocuous toxoid

Neutralization of circulating antitoxin by toxoid   A specimen of purified toxoid prepared by Dr L Holt was available, containing 1700 Lf per mg N and 250 Lf per mg ; its M R D was 10 Lf   It readily formed a clear solution

in 0 85 per cent saline   Its equivalence with the antitoxin used throughout the experiments was checked by constant-antigen (Ramon) titrations, and solutions were made containing 40, 80, 400 and 1200 Lf per ml   The antitoxin was used at 40 u per ml   Circulating toxoid *per se* did not affect intradermal toxin. Toxin was titrated in 9 guinea-pigs , five minutes after the injection of the toxin three received 1 5 Lf toxoid per kg , intravenously, and three received 15 Lf per kg   The dose-response curves from all three groups were linear, parallel and substantially coincident.
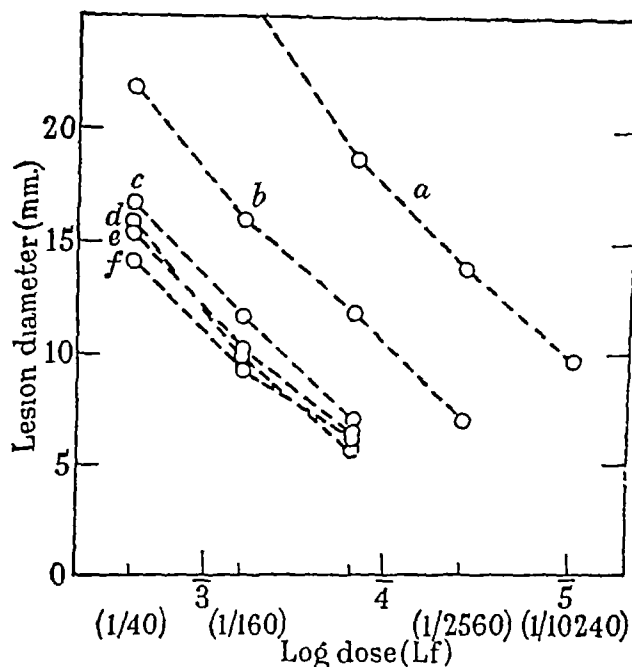

Fig 10 —The effect of intravenous toxoid on the indirect neutralization of intradermal diphtheria toxin by circulating antitoxin
    (*a*) Antitoxin *nil*, toxoid *nil*
    (*b*) Antitoxin 60 u per kg , ten " equivalents " of toxoid after 2 minutes
    (*c*) As (*b*), but the toxoid after 1 hour
    (*d*) and (*e*)   As (*b*), but the toxoid after 2 and 3 hours
    (*f*) Antitoxin alone

The " deviation " of circulating antitoxin by toxoid was tried in guinea-pigs given 60 u antitoxin per kg , and intradermal toxin 3 to 5 minutes later   Two minutes after the toxin either 1, 2, 10 or 30 equivalents of toxoid were injected intravenously   In other tests, the toxoid and antitoxin were mixed *in vitro* before injection   The results were as follows
    One or two equivalents of toxoid given 2 minutes after toxin slightly diminished the efficacy of the indirect neutralization , ten equivalents diminished it significantly (Fig 10*a*, *b* and *f*), but 30 equivalents were no better   The curves *c*, *d* and *e* in Fig 10 show the decrease in the toxoid effect with increasing delay in injecting it after the toxin
    At first sight these results are compatible with Friedemann's hypothesis, because one equivalent, presumably neutralizing all circulating antitoxin, did not affect the skin toxin appreciably, and the ten equivalents may have done so by passing from the circulation into the tissues and disturbing the toxin-

antitoxin complexes already formed there (cf the modifying effect of toxoid added to toxin-antitoxin mixtures *in vitro* , Schmidt and Scholz, 1929) There is, however, good evidence that the one equivalent of toxoid did not neutralize all the circulating antitoxin Thus, 10 equivalents of toxoid diminished the efficacy of antitoxin to the same extent when given just *before* the toxin, and when mixed with antitoxin *in vitro*, and injected either immediately, or after standing for 1 hour at 37° C. That is to say, the combination between 10 equivalents of toxoid and antitoxin, whether taking place *in vivo* or *in vitro*, was incomplete in the sense that some antitoxin was available *in vivo* for the neutralization of tissue toxin Moreover, since the degree of neutralization was the same in all cases, the effect of toxoid before or after toxin was the same as the injection in the first place of a smaller amount of antitoxin We must therefore conclude that, owing perhaps to slowness of combination and differences in the rates of removal of toxoid and antitoxin from the circulation, one equivalent of toxoid did not neutralize *in vivo* , and that 10 equivalents acted by neutralizing the circulating antitoxin, and not by penetrating the tissue and modifying any toxin-antitoxin complexes formed therein

Reading off the 14 mm lesion-diameters in Fig 10, the toxic doses in Lf for no antitoxin (*a*), antitoxin + toxoid (*b*) and antitoxin alone (*f*) are 1/2500, 1/320 and 1/40 In this instance the toxoid has diminished the antitoxic potency by half , so that at least half the neutralization in the skin lesion took place *after* the injection of toxin.

DISCUSSION

The neutralization of intradermal toxin is only remotely analogous to antitoxin therapy of natural diphtheria Nevertheless, the skin lesion is peculiarly apt for investigation of the problems of antitoxin therapy It is surprisingly regular, and the regression line of log dose upon lesion-diameter is linear and provides a measure of antitoxic efficacy whose statistical significance can readily be assessed The method is a logical extension of the " multiple Schick test " of Glenny, Hopkins and Pope (1924) , in addition to the end-point determined by such a titration the dose-response curves yield numerical information about the fate of the incompletely neutralized dose of toxin, as well as about the neutralized dose The end-point is in fact a limiting case , but without information about the conditions of which it is a limit, it cannot be considered representative of the toxin-lesion in general I do not suggest that all diphtheria immunity should be measured by the response to graded doses of toxin, but the method deserves consideration when matters of special immunological interest are in question

Friedemann based his conception of the rapidly-established definitive concentration of tissue antitoxin upon the following arguments

(1) Intradermal toxin is neutralized by circulating antitoxin in constant proportions It cannot therefore have combined with the tissues

(2) The proportionality is disturbed when the antitoxin is given as little as two minutes after the toxin Toxin and antitoxin must, therefore, combine within at most two minutes

(3) The degree of indirect neutralization is the same when the antitoxin is given two minutes and 3 hours before the toxin The tissue concentration

of antitoxin must therefore be constant over a period of hours, and rapidly established, at least in a molecular zone surrounding the susceptible cells

(4) It follows that the ratio of indirect and direct neutralizing doses of antitoxin (Ai Ad) is a valid measure of the relative concentration of antitoxin in the blood capillaries and the tissues

I have confirmed Friedemann's findings in (1) and (3) but not in (2)　The disturbance of proportionality, and any difference. in the nature or degree of neutralization by antitoxin given a few minutes before and after toxin, are undetectable when small doses of toxin are used　It does not therefore follow that all effective combinations of toxin and antitoxin must take place within a few minutes

With regard to (1), Friedemann's conclusion is not inevitable　Thus, if tissues and antitoxin competed for toxin, if fixation were proportional to the concentration of toxin, and if reversal of tissue-intoxication were proportional to the concentration of antitoxin continually passing from the circulation to the tissues, proportionality might hold equally well

The deduction from (3) may be correct, but the equilibrium so established is not necessarily definitive in the sense that the resulting concentration of tissue antitoxin at the moment of injecting toxin finally determines the degree of neutralization　It is highly unlikely that in the normal animal the equilibrium is static, because, whether antitoxin is fixed to the tissues or not, when the tissues are saturated, excess antitoxin will be removed *via* the lymphatics and its place taken by antitoxin from the blood (Drinker and Yoffey, 1941)　But if the equilibrium is dynamic, then the tissue concentration at the moment of injecting the toxin can be definitive only if the toxin is rapidly fixed to the tissues, and reversal of the fixation by antitoxin is impossible　As the dose-response to toxin shows, toxin is rapidly *absorbed* to the tissues, otherwise the lesion-diameters made by any dose of toxin above a certain concentration would tend to equal the limits of spread of the injection fluid, it is not, however, rapidly *fixed*, for fixation that is irreversible by saline in a 2-hour-old lesion may to some extent be reversed by antitoxin in lesions up to 6 hours old

Friedemann's contention that the tissue. antitoxin is confined to a protective molecular zone round the cells, though it serves to explain the rapid establishment of equilibrium, does not affect objections to the hypothesis based on the demonstration of competition　It is far more likely that intradermal toxin is neutralized progressively by antitoxin continuously passing across the capillary endothelium　The transendothelial flow may be constant in the normal animal There is, moreover, no evidence that during the first five hours of a lesion when the fixation of toxin takes place, diphtheria toxin induces a pathological increase in capillary permeability as the α-toxins of *Cl welchii* and *Staph aureus* do, though after two hours the flow of antitoxin may be accelerated by a physiological response of the skin vessels

The moment the flow of antitoxin ceases to be effective will depend on the rate of fixation of toxin to the tissues, the concentration of antitoxin entering the tissues and the relative avidities of tissues and antitoxin for toxin　Diphtheria toxin is a slowly-acting capillary poison, though when it is injected into passively immunized animals together with substances that increase capillary permeability, the efficacy of the circulating antitoxin is greatly increased (Friedemann, Traub and Langstadt, 1946)　The strikingly low efficacy of indirect

neutralization of tetanus toxin (Friedemann, Zuger and Hollander, 1939c) might well result from an even lesser toxicity for capillary endothelium, and the notoriously rapid fixation of toxin to neural tissue

The inaccessibility of diphtheria toxin in the tissues to circulating antitoxin is at first mechanical, then pharmacological The shortest period after which late intravenous injection of antitoxin will fail to neutralize intradermal toxin is important in antitoxin therapy but it is not a valid measure of the rate of fixation of toxin The fact that intravenous antitoxin is largely ineffective given after 2 hours does not mean that the 2-hour-old lesion cannot be affected, but that the summation of antitoxin passing into the intoxicated tissue necessary for neutralization is not reached in a certain critical time, $(2 + x)$ hours The value of x is uncertain, but the results of superinjecting antitoxin suggest that x is greater than 1 hour, but not much more than 4 hours

Though a rigorous experimental disproof of Friedemann's hypothesis is wanting, the demonstration of competition in the tissues and all that it implies, contradicts some of its postulates , and the analyses of the dose-response to toxin both alone, and directly and indirectly neutralized, are difficult to reconcile with it It follows that in the absence of data about rates of toxin fixation and transendothelial passage of antitoxin, and about the reversibility of the tissue-toxin combination, Friedemann's Ai Ad ratio cannot yield any precise information about the equilibrium values of the concentration of antitoxin inside and outside the blood vessels of the normal animal , and in any event, the Ai Ad ratio is formally invalid, since its magnitude depends on the response level chosen for intersecting two statistically heterogeneous dose-response lines

SUMMARY AND CONCLUSIONS

When diphtheria toxin is injected intradermally into a guinea-pig it is absorbed immediately to the skin tissues A concentration gradient is thus established, declining outwards from a central maximum value depending on the dose of toxin, such that the diameter of the resulting inflammatory lesion at 24 hours is proportional to the logarithm of the dose of toxin injected The resulting dose-response curve provides a measure of immunity that is more informative than the end-points determined by the usual methods of titration of toxin, and is susceptible to precise statistical analysis

Immediately after injection the toxin is lightly held by the skin-tissues and can be washed away by saline Within 2 to 3 hours all the toxin is fixed too firmly to be removed by saline, but can still be neutralized to some extent by antitoxin That is to say, there is a degree of combination of toxin and tissue that can be reversed by antitoxin Antitoxin was not demonstrably effective after 5 to 6 hours

During the first six hours, fixation that is irreversible by antitoxin proceeds at an approximately uniform rate , there is little evidence that in this time the toxin induces any pathological increase in the permeability of the blood capillaries

It appears that in the normal guinea-pig, intravenously injected antitoxin passes at a regular rate through the tissues into the lymphatic system, and there is thus established a dynamic equilibrium across the capillary endothelium The equilibrium concentration in the tissues at the time of an intradermal dose

of toxin neutralizes only part of that dose , at least half may be neutralized by antitoxin passing into the tissues after the injection of toxin

The moment at which circulating antitoxin ceases to be effective is therefore dependent on the rate of its passage across capillary endothelium, and the rate of fixation of toxin to the tissues   In the indirect neutralization of intradermal toxin, the moment occurs 2 to 3 hours after the injection of toxin   Since, however, the time interval is the resultant of two opposing rates, it is not a valid measure of the rate of fixation of toxin , and for the same reason, the ratio of the amounts of antitoxin neutralizing a standard dose of toxin by the indirect route and by direct mixing *in vitro*, is not a valid basis for estimating the equilibrium values for the concentration of antitoxin inside and outside the blood capillaries of the normal animal

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THE NATURE OF ANTISTREPTOLYSIN " S " IN THE SERA OF MAN AND OF OTHER SPECIES · ANTISTREPTOLYSIN TITRES IN NORMAL AND DISEASED STATES

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THE study of haemolysins produced by streptococci and of antibodies against them was greatly clarified by Todd (1938), who first clearly differentiated streptolysin " S," a serum-soluble, oxygen stable haemolysin, from streptolysin " O," which is readily formed in serum-free media and is inactivated by oxygen   It has been shown by him (Todd, 1932), and by many workers since, that streptolysin " O " is a powerful antigen, and that infection with strains of streptococcus which produce this haemolysin is generally followed by a sharp rise in the antistreptolysin " O " content of the serum   Streptolysin " S," on the other hand, does not readily give rise to antibodies, and although sera from various animals have long been known to contain inhibitors of " streptococcal haemolysin " (Lyall, 1914 , McLeod and McNee, 1913), no increase in inhibitory action was observed after injection of haemolytic streptococci   Todd, however, claimed that by prolonged courses of intravenous injections of living Group A streptococci it was possible to obtain antisera in rabbits to streptolysin " S," that such antisera were specific for streptococci of this group, and that antibodies against streptolysin " S " were distinct from those against streptolysin " O " (Todd, 1938, 1939 , Herbert and Todd, 1944)   In 1939 Todd, Coburn and Hill published a study of the antistreptolysin " S " and " O " titres in normal adults, and in children with haemolytic streptococcal infections with and without rheumatic fever   The variations in antistreptolysin " S " titre were not large, but from a statistical analysis of their data these authors concluded that in response to infection with haemolytic streptococci there is a rise in the antistreptolysin " S " titre considerably above the normal level in the sera of all groups studied, with the exception of those children who developed clinical signs of rheumatic activity   Furthermore, during rheumatic attacks the antistreptolysin " S " titres tended to be lowest when the clinical symptoms were most pronounced

Since antibodies to other streptococcal products (e g   streptolysin " O," streptokinase, hyaluronidase, proteinase) have been shown by other workers to be increased in the sera of patients with rheumatic fever in much the same way as in the sera of persons with streptococcal infections, but without clinical rheumatic activity, it seemed that the antistreptolysin " S " response might be a clue to some significant difference between the rheumatic and the non-rheumatic groups   A limited study of such sera, which is reported below, showed that nearly all sera taken from sick persons had lower anti-streptolysin " S " titres

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than those from normal people, and failed to reveal the difference found by Todd, Coburn and Hill (1939) between those streptococcal infections which were accompanied by clinical rheumatic fever and those which were not   The investigation was extended therefore to attempts to produce immune sera to streptolysin " S " in rabbits and a horse, both by the injection of living streptococci and by potent filtrates of yeast nucleic acid broth cultures (Okamoto, 1939)   These attempts were not successful, and since an examination of sera from several species of animal revealed considerable amounts of antistreptolysin " S " in cases where any streptococcal infection could be excluded, the status of antistreptolysin " S " as an antibody in the accepted sense of the word is called in question   In this paper are presented the results of antistreptolysin " S " investigations in untreated sera

MATERIALS AND METHODS

Sera

Sera were obtained fresh and were stored without preservative at − 20° C No alteration in antistreptolysin titres were observed during several months under these conditions

Streptolysin ' S "

Two strains of Group A streptococcus were used for production of streptolysin " S "   During the earlier stages the strain used (NY5) was a Type 10 streptococcus, which produced powerful streptolysin " S " and some streptolysin " O "   It was grown at 37° C overnight in a beef heart infusion broth containing 10 or 20 per cent horse serum   The cocci were centrifuged down and were extracted with horse serum, thereby giving a powerful haemotoxin, which was stored in ampoules at − 76° C

During the later stages there became available a Type 11 strain (Blackmore) which had previously been shown by Todd to produce streptolysin " S " but not " O "   This strain was grown overnight at 30° C in 20 per cent serum broth, and the euglobulin fraction of the supernatant fluid, which contained the bulk of the streptolysin activity, was precipitated according to the method described by Herbert and Todd (1944)   The precipitate was dissolved in borate buffer saline pH 8 0, and stored in ampoules at − 76° C   At this temperature both the above preparations were stable for at least 6 months, although at − 20° C or higher they rapidly declined in potency

Towards the end of this investigation it became desirable to have a preparation of streptolysin " S " free from serum, in order to eliminate possible effects of the serum moiety   Use was made of the observation of Okamoto (1939), recently amplified by Bernheimer and Rodbart (1948), that in the presence of yeast nucleic acid large amounts of oxygen stable streptolysin are found even in the absence of serum   Strain Blackmore grown for 24 hours in infusion broth containing 1 per cent yeast nucleic acid yielded a potent haemolytic filtrate, which was reasonably stable at − 20° C or at 4° C   As will be shown below, this filtrate was serologically equivalent to haemolysin produced in serum broth

Finally, for comparison, a strain of group C streptococcus (Loewenthal M) was grown in 20 per cent horse serum broth in the same manner as the Group A Type 11 strain, and from it the haemolytic euglobulin fraction was also prepared

Titration of antistreptolysin " S "

The method used was that given by Todd (1938), in which the unknown serum is titrated against a sample of streptolysin which has been standardized against a standard serum at the time of test   The estimations were carried out in borate buffer saline, at pH 8 0, and rabbit erythrocytes were used as indicators It was found that a suitable amount of streptolysin was that which caused 50 per cent lysis of the erythrocytes in 2 hours under standard conditions   A normal human serum was set aside as a standard , it was kept stored at − 20° C To it was assigned an arbitrary value of 10 units antistreptolysin " S " per ml Although this standard was not the same as Todd's standard, it was probably fairly close, since the range of antistreptolysin titres found did not differ greatly from his previous records

Titrations of antistreptolysin " O "

These were done by Todd's (1938) method, modified by use of an isotonic phosphate buffer pH 6 5 for dilutions and of M/100 thiolacetic acid for reduction as recommended by Herbert and Todd (1941)

Immunization of experimental animals

Rabbits —Six rabbits of mixed breeds were given intravenous injections of Streptococcus Blackmore grown in 20 per cent rabbit serum broth, and suspended in the supernatant fluid   The first two injections consisted of killed organisms, but all later injections contained living streptococci   Injections were given 2 or 3 times weekly, with rest periods of a fortnight or more between courses   The total volumes of packed living organisms injected varied from 0 6 to 1 0 ml , and of culture fluid from 12 to 20 ml   Serum samples were taken at the beginning, in the middle, and one week after the end of each course

Horse —The course of immunization was kindly performed by Dr C L Oakley, at the Wellcome Research Laboratories, Beckenham   Five intramuscular doses of formolized culture filtrate from streptococcus Strain Blackmore grown in yeast nucleic acid broth were followed by a total of nearly 3 l of untreated filtrate, in 26 doses over a period of 15 weeks   Test bleedings were made weekly

RESULTS

Antistreptolysin " S " in human sera

The summarized results of estimations on pathological and normal human sera are given in Table I, from which it appears that the highest antistreptolysin " S " titres were found in normal adults and in late convalescent cases of rheumatic fever   During the acute phases of streptococcal and other infections, and during the early stages of convalescence, the antistreptolysin " S " titres were comparatively low   It is also apparent that antibodies against streptolysins " S " and " O " behaved independently

The lowest antistreptolysin titre encountered (not included in the table) was 2 7 units/ml in the serum of a patient with steatorrhoea who was on a fat-free diet, and the highest value was 15 units/ml in the serum of a patient with Type II nephritis and raised serum lipoids

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TABLE I —*Antistreptolysin " S " Titres (units/ml) in Human Sera*

The mean antistreptolysin " O " titres are given for comparison

| Disease | No of sera | Anti-streptolysin " S " | | Anti streptolysin " O " |
		Range	Mean	Mean
Scarlet fever—acute .	8	3 2–8	6 3	35
Convalescent (14–16 days)	8	4–8	6 2	240
Streptococcal pharyngitis—acute	4	4–6 7	5 2	410
Subacute and convalescent	4	2–9 5	6 1	240
Rheumatic fever—acute	8	5–6 7	6 3	428
Subacute	16	4–10	6 7	261
Convalescent	15	5 3–12 5	8 4	275
Rheumatoid arthritis—acute	4	5 3–9 5	6 5	92
Subacute	4	4–7 8	6 3	130
Normals	14	8–12 5	9 7	48

Antistreptolysin " S " in the sera of other animals

In Table II are given antistreptolysin " S ' titres of various mammals, of representative teleost and elasmobranch fishes and of a marine invertebrate The marine animals were included because they are not known to be subject to streptococcal infections, and it was of interest to learn whether their sera would inhibit streptolysin " S " The high titres found in normal rat and guinea-pig sera are noteworthy

TABLE II —*Antistreptolysin " S " in the Sera of Various Animal Species*

| Species | No of sera | Antistreptolysin " S " (units/ml) | |
		Range	Mean
Normal human .	14	8–12 5	9 7
Horse	7	10–27	16
*Horse (Cl welchii antitoxin)	6	5–7	5 5
Rabbit	12	3 2–8 1	6 6
Rat	2 pools (36 sera)	50–60	55
Guinea-pig	4 pools (12 sera)	32–56 .	43
Ox	1		23
Sheep	1		12
Plaice (*Pleuronectes*)	2 pools	48–50	49
Dog fish (*Scyllium*)	1 pool		5
Spider crab (*Carcina Maia*)	1 pool (whole blood)		1 2

* These sera were preserved with 0 3 per cent tricresol It was found that this preservative caused a fall of 25 per cent in the antistreptolysin " S " titre in one week at + 4° C

Correlation between antistreptolysin " S " titres using different sources of streptolysin " S "

Duplicate estimations were made on a number of sera, both of human and of other species, covering a wide range of antistreptolysin titres, in order to determine whether significant differences in titre would be obtained when streptolysin " S " from different sources was used   The results (Table III) showed that no significant differences were found between streptolysins prepared in different ways from Group A streptococci, or between streptolysins from a strain of Group A and a strain of Group C streptococci

The apparent serological identity between yeast nucleic acid broth streptolysin and serum broth streptolysin is interesting, since in chemical properties the partially purified streptolysins, although superficially different, both appear to have properties of lipo-nucleoproteins

Immunization experiments

The results of the rabbit experiments are given in Table IV   No significant increase in antistreptolysin " S " titres was observed   During periods when the rabbits were seedy owing to the injections, and were losing weight, the titres tended to be lower than during rest periods   That the animals were capable of producing antibodies was shown by measurement of the streptococcal agglutinins, which reached titres of more than 1 100,000   It is of interest to record, in confirmation of Herbert and Todd (1944), that the Strain Blackmore did not cause a rise in antistreptolysin " O," and that presumably not even traces of streptolysin " O " are elaborated by this organism

In the horse the antistreptolysin " S " titre rose from an initial value of 9 units/ml to 20 units/ml , but at the end of the course it was only 5 units/ml These titres lie within the range found for normal horses, though the last is unusually low

<div align="center">DISCUSSION</div>

An antibody in the commonly accepted sense of the term is a serum component which combines with the antigen and is produced in response to the introduction of the antigen into the tissues of the organism   It is a matter of observation that antibodies are proteins, and that they are found in the β- or γ-globulin fraction of the serum   In the investigation reported antistreptolysin S failed to conform to this definition of antibodies   It was found to occur in high titre in the sera of normal animals, in the complete absence of any evidence of either present or past streptococcal infection   It could not be produced either in rabbits or in a horse by intensive courses of " immunization " with streptolysin S   Furthermore, as will be shown in a subsequent publication, the antistreptolysin S activity of serum is not predominantly associated with the β- or γ-globulin fractions   For these reasons it is considered that antistreptolysin S is not a true antibody   It may well be associated with a normal constituent of serum which fluctuates in amount irrespective of streptococcal infection   An analogous situation is presented by the non-specific substances in many sera which inhibit the influenza virus haemagglutination reaction, and which fluctuate from time to time without known cause (Smith and Westwood 1949)   If individual fluctuations depend in some degree upon metabolic activity, illnesses of different types, including streptococcal infections, might be expected to produce similar

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TABLE III—*Correlation Between Antistreptolysin Titres of Sera when Assayed Against Streptolysin " S " from Different Sources*

	Source of streptolysin	No of sera	Nature of sera	Antistreptolysin (units/ml) Range	Mean	S D of difference
(1)	Euglobulin ppt from Group A, Type 11	25	Human	4–12.5	7.34	0.31
	Serum extract Group A, Type 10	25	,,	4–11.5	7.33	
(2)	Euglobulin ppt from Group A, Type 11	14	Human and other animals	2.5–50	12.8	1.8
	Yeast nucleic acid broth filtrate from Group A, Type 11	14		2–56	13.9	
(3)	Euglobulin ppt from Group A, Type 11	11	Human and rabbit	4–25	11.2	1.4
	Euglobulin ppt from Group C	11		3–25	12.6	

TABLE IV —*Antistreptolysin " S " Titres in Sera of 6 Rabbits Given Repeated Intravenous Injections of Living Hæmolytic Streptococci*

	Antistreptolysin " S " (units/ml)		Mean weight (kg)
	Range	Mean	
Preliminary	5–8 1	6 1	3 0
Middle of first course	2 7–3 2	3 1	2 7
End of first course	2 9–4 3	3 6	2 8
„ second course	3 1–6 0	4 9	2 7
„ third course	2 5–6 7	5 0	2 9

effects on antistreptolysin S levels, but the degree might depend upon the stage, activity and duration of the illness   This could account for the observation of Todd, Coburn and Hill (1939) on sera from cases of rheumatic fever, and our own failure to confirm any correlation between antistreptolysin S level and type of disease

The present work failed to provide any explanation of Todd's results (1938), in which he obtained evidence of an immune response to streptolysin S in rabbits by the inoculation of living streptococci

SUMMARY

Antistreptolysin " S " titres have been measured in sera from human beings with streptococcal infections with and without rheumatic fever, from various mammals and from representative marine animals

The level of antistreptolysin " S " was not specifically correlated with streptococcal infection

No evidence was obtained of increase in antistreptolysin titre after prolonged immunization of rabbits and a horse with living haemolytic streptococci or haemolytic culture filtrates

Streptolysin " S " produced in serum broth is serologically indistinguishable from streptolysin produced in yeast nucleic acid broth without serum

It is suggested that antistreptolysin " S " is not normally an antibody in the accepted sense of the word

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THE EFFECT OF SEVERAL DILUENTS ON PROTHROMBIN ACTIVITY CURVES

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WITH the increased use of anticoagulant drugs more attention has been focused on blood coagulation methods, especially the prothrombin test   Many variations in performing the test and computing the results have been proposed, which have tended to confuse proper evaluation of the results   Several attempts, however, have been made to standardize prothrombin test procedures, and these have shown Quick's one-stage method to be accurate and easily adaptable to routine testing (Aggeler, Howard, Lucia, Clark and Astaff, 1946 , Fisher, 1947, 1948 , and Martin, Curfman and Cavano, 1947)

Several modifications of Quick's method are used in which the plasma is diluted to either 50 or 12 5 per cent before performing the test   The use of diluted plasma and its sources of error have been discussed in previous communications (Fisher, 1947, 1948)   However, some laboratories prepare serial dilutions of normal plasma in order to standardize their thromboplastin preparations   This is not necessary when Quick's formula for the hyperbolic curve is used (Fisher, 1947, 1948)

The use of these hyperbolic " prothrombin activity curves " for standardizing thromboplastin reagent has led to some discussion as to the nature of the plasma diluent which should be used   Physiologic sodium chloride solution (0 9 per cent) is used most widely   However, this practice has been criticized by those who feel that prothrombin-free plasma is the only proper diluent   The present study was made, therefore, to note the effect of several different diluents for plasma in the preparation of these activity curves

PREPARATION OF REAGENTS

(1) Triple distilled water

(2) Physiologic saline solution (0 9 per cent NaCl, reagent grade)

(3) Normal blood serum   Blood was drawn by venepuncture and allowed to clot in the tube   It was then centrifuged at moderate speed for 30 minutes and the supernatant serum withdrawn for use

(4) Protein-free filtrate of blood serum   The serum proteins were precipitated with tungstic acid (Levinson and MacFate, 1946) and the solution was filtered   The filtrate was neutralized to approximately pH 7 with $N/10$ sodium hydroxide   Control   The protein-free filtrate prepared in this manner gave a negative biuret test, as compared to a portion of the original serum, which gave a positive biuret test

(5) Adsorbed (prothrombin-free) plasmas

(a) Barium plasma   The prothrombin was adsorbed on a 30 per cent suspension of barium sulphate, according to the method suggested by Tuft and Rosenfield (1947)

(b) Alumina plasma   A first preparation was made by the general formula of 1 volume of commercially prepared aluminium hydroxide gel to 8 volumes of citrated plasma   It was incubated for 15 minutes at 37° C , then centrifuged, and the supernatant plasma drawn off for use   This preparation was unsatisfactory, as evidenced by a preliminary test applied to all of the adsorbed preparations before use   One drop of the adsorbed plasma to be tested was placed on a glass slide and one drop each of thromboplastin and calcium chloride were added   If the fluid clotted (usually within twenty seconds), the prothrombin had not been adsorbed

A different alumina preparation was then prepared, according to the method of Quick (1935), using a thicker aluminium hydroxide cream   This latter plasma was acceptable for use

(c) Magnesium plasma   was suggested   However, recent work by Maltaner (1948) has shown that the presence of magnesium ions enhances the rapidity of clotting at lower concentrations of calcium ion   Therefore it was considered not desirable to add these ions to the preparations, especially when dilution would decrease the level of calcium ions

Tocantins (1944) has shown that some of the prothrombin and fibrinogen can be adsorbed on asbestos fibres, leaving the concentration of antithrombin unchanged

(6) Plasma   Blood was drawn from normal healthy dogs and oxalated in the proportion of 1 part of M/10 sodium oxalate to 9 parts of blood   The blood was centrifuged, and the supernatant plasma used as the source of prothrombin-containing plasma for the tests

(7) Thromboplastin was prepared from acetone-dehydrated rabbit brain (Difco), and M/40 calcium chloride was used to recalcify (Fisher, 1948)

EXPERIMENTS

Barium plasma was selected as representative of the adsorbed plasmas because of the excellent results reported by Tuft and Rosenfield (1947)   Quick (1935) has found alumina plasma to be consistently good   Magnesium plasma was considered to be not desirable, as stated above

All reagents were freshly prepared and stored in the ice box until used   They were then allowed to come to room temperature   Dilutions of the normal oxalated dog plasma were made, with each diluent, in the order of 100, 70, 50, 40, 30, 20 and 10 per cent   Two determinations by Quick's method (1940) were made at each dilution on each sample of plasma   The average of the two determinations was then taken, and the figure plotted (Fig 1)

DISCUSSION

The prothrombin time for undiluted plasma was much shorter than is usually encountered with human plasma, since dog plasma contains a greater concentration of prothrombin (Quick, 1935)   When normal dog serum was used as a
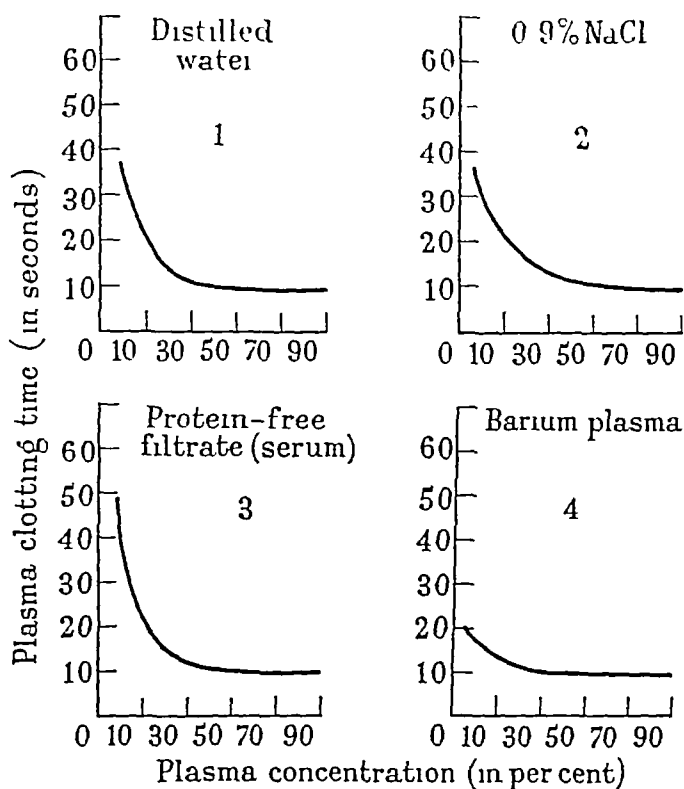
FIG 1

diluent, the diluted preparations were clotted within the original tubes before they could be used   This was due to the presence of small but sufficient amounts of calcium ions, prothrombin, fibrinogen and activator substances in the serum   Preparations made with barium sulphate-adsorbed plasma gave shorter prothrombin times and a somewhat more flat curve than the others   This was believed to be due to incomplete adsorption of the prothrombin, as well as the presence of other protein and ionic substances   If barium sulphate plasma is to be used, the technician must be sure that it is a pure, thick preparation and that adequate adsorption has been obtained   Owren (1947) has shown that addition of prothrombin-free (adsorbed) plasma enhances the coagulability of pathological plasmas   Saline solution, distilled water, and protein-free filtrate of serum gave results which were all similar   The characteristic hyperbolic curve was obtained in all cases   The times obtained with these preparations were all within the mean standard deviation for each point on the dilution curve, as previously calculated (Fisher, 1947)

CONCLUSIONS

From the results obtained in these experiments it is seen that the essential change produced by serial dilution of normal plasma is reduction in the amount of prothrombin present   Distilled water 0 9 per cent saline solution and protein-free filtrate of normal serum produced uniformly similar results as diluents, duplicating the characteristic hyperbolic curve   Prothrombin-free (adsorbed)

plasmas may be sources of error if a good preparation is not made Owren (1947) has shown that addition of prothrombin-free plasma to normal plasma shortens the prothrombin time by Quick's method Normal serum could not be used as a diluent due to the presence of small amounts of prothrombin, fibrinogen and activator substances

Nitshe, Gerarde and Deutsch (1947) and Owen and Bollman (1948) recommend 0 3 per cent fibrinogen solution as a diluent, claiming that the end point is thereby made sharper at the higher dilutions. This would be favourable at the lower concentration end of the curves, or if only 12 5 per cent plasma were being used However, the addition of extra amounts of proteins and neutral salts to whole plasma can only serve to upset the delicate colloidal balance between the reactants of the blood coagulation mechanism

SUMMARY

An experimental study has been presented in which was shown the effect of several diluents on "prothrombin activity curves" Normal serum was shown to be a poor diluent due to the presence of small amounts of prothrombin, fibrinogen and activator substances Adsorbed plasma may be a source of error if a proper preparation is not made Distilled water, 0 9 per cent sodium chloride solution and protein-free filtrate of normal serum showed uniformly consistent results, reproducing a smooth hyperbolic curve

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NITRATASE ADAPTATION   AN APPARENT EFFECT OF PENICILLIN WHICH CAN BE REVERSED BY SALT

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GRAM-POSITIVE bacteria concentrate certain amino-acids in the free state in the internal environment prior to metabolism (Gale, 1947, Taylor, 1947) The passage of amino-acids across the cell-wall into the cell may be by a process of diffusion, as in the case of lysine, or may require energy drawn from exergonic metabolism, as in the case of glutamic acid   Gale and Taylor (1947) have shown that the assimilation of glutamic acid by *Staph aureus* is blocked if the cells grow in the presence of penicillin   Taylor (1947) was unable to demonstrate the presence of free amino-acids within Gram-negative cells, and there is as yet no knowledge of the nature of amino-acid assimilation in such organisms, or whether the processes involved are affected by penicillin   Pollock and Wainwright (1948) showed that the rate of adaptation to nitrate of a coliform organism was markedly influenced by the nature of the nitrogen source available   The formation of the adaptive nitratase was increased by the addition of amino-acids, a variety of amino-acids being more effective than a single one, and maximal adaptation occurring when a protein hydrolysate was supplied as N-source It is clear that the rate of nitratase production could be used as an indirect measure of the assimilation of amino-acids   This work was accordingly undertaken in an attempt to study some properties of amino-acid assimilation in this Gram-negative organism and to investigate the action of penicillin on the assimilation   It is shown below that, although an effect of penicillin on adaptation was discovered, it is probable that this action is not related to amino-acid assimilation or protein synthesis   The results are inexplicable in the present state of knowledge, but are recorded since they may be related in general to studies of penicillin action

ORGANISM AND METHODS

The organism used was the coliform " 1433 " used by Pollock and Wainwright (1948)   It was maintained on casein-digest-agar slopes   The organisms used in experiments quoted below were grown on the surface of casein-digest agar for 16 hours at 30° C   The organisms were harvested, washed and suspended in a salt solution of the following composition   3 0 g KH_2PO_4, 10 0 g Na_2HPO_4 3 0 g NaCl 2 1 g $MgSO_4$, $7H_2O$, 1000 ml distilled water

Methods used for the estimation of nitrite, nitratase activity and for following adaptation to nitrate were as described by Pollock (1946) and Pollock and Wainwright (1948)   In principle, nitratase is estimated by determination of nitrite formed from nitrate in the presence of the organisms, with sodium formate as

H-donator, adaptation to nitrate is followed either by estimation of nitrite formed during incubation of the organism with nitrate, glucose and an external N-source or by estimation of the nitratase activity of the organisms before and after such adaptation. All incubations were carried out anaerobically at 37° C, and quantities of reagents were the same as those used by Pollock and Wainwright (1948).

The penicillin used was a commercial crystalline preparation assayed at 1640 Oxford Units per mg. The vitamin-free casein hydrolysate used was obtained from Ashe Laboratories.

Preliminary investigations

The adaptation towards nitrate of cells grown in nitrate-free medium was first followed by estimation of the nitrite formed when the cells were incubated in the presence of nitrate and glucose. The acceleration of adaptation by the addition of ammonia or mixtures of amino-acids was confirmed. The effect of the presence of penicillin was tested in systems to which vitamin-free casein-hydrolysate was added as external N-source. No effect on adaptation was observed with penicillin concentrations up to M/5500 (c 100 units per ml), when concentrations greater than this were present the rate of adaptation began to fall, in comparison with penicillin-free controls, after 90 to 120 min. Since no effect could be observed under 90 min it seemed probable that some degree of growth must occur in the cells before penicillin exerts any inhibitory effect. The action then takes place under conditions similar to those reported for the action of penicillin on growth, respiration and glutamic acid assimilation in *Staph aureus* (Chain and Duthie, 1945, Gale and Taylor, 1947). The effect of treating the cells with penicillin before adaptation was next tested. The cells, directly after harvesting, were suspended in the salt solution (final suspension strength = 0 25 mg dry weight of cells per ml) containing M/100 NH₄Cl and M/100 glucose. Penicillin was added to various samples at concentrations ranging from 10 to 1000 units per ml and the cultures then incubated at 37° C for 2 hours. The cells were centrifuged down, washed once with salt solution and then resuspended in salt, nitrate, glucose and casein hydrolysate solution for adaptation. The course of nitrite production showed that pretreatment with 10 units penicillin per ml resulted in a slight but definite acceleration of the rate of adaptation, whereas pretreatment with 100 to 1000 units penicillin per ml resulted in a marked decrease in the rate of adaptation. Parallel experiments were carried out in which the casein hydrolysate was omitted during adaptation, and it was found that the course of nitrite production was apparently inhibited in the same proportion as in the presence of casein hydrolysate. It was difficult to gauge the relative inhibitory effects in the various media by this method, and further experiments were made by estimating the nitratase in the washed cells before and after adaptation.

General procedure

The cells were washed from the surface of agar in c 10 ml salt solution, centrifuged down and resuspended in salt solution to a final strength of 1 0 mg dry weight of cells per ml. To v ml of suspension were then added $3x$ ml salt solution containing M/100 NH₄Cl, M/100 glucose and penicillin of the required

concentration   The suspensions were incubated for 2 hours at 37° C , centri-
fuged down and washed once in $4x$ ml salt solution   The washed cells were
then suspended in x ml salt solution and added to $3x$ ml salt solution containing
$M/100$ NaNO$_3$, $M/100$ glucose and 0 1 per cent casein hydrolysate or other
N-source as described below   A sample of unadapted cells was tested for nitratase
activity, and the remainder incubated for 90 min at 37° C in an atmosphere of
nitrogen   The cells were then centrifuged down, washed once in $4x$ ml water
and their nitratase activity determined   Until the final stage the cells were
kept in salt solution and, except when on the centrifuge, at 37° C   All experiments
were controlled by estimations on cells treated in the same manner, but with the
omission of penicillin during the first period of incubation

<div align="center">RESULTS</div>

<div align="center">*Inhibition of Nitratase Formation by Penicillin*</div>

Effect of penicillin concentration

Incubation of non-adapted cells with glucose and nitrate results in an increase
of nitratase activity even in the absence of an added source of nitrogen   The
effect of pretreatment with penicillin was to reduce the rate of this increase
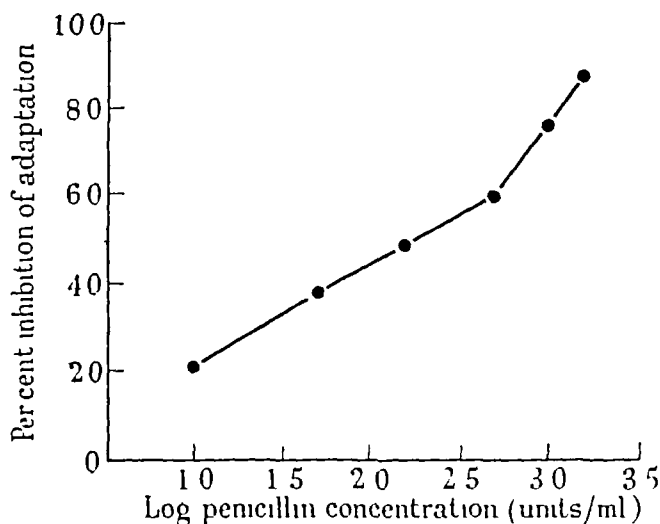Fig 1 shows the degree of inhibition obtained by pretreatment with various

Fig 1 —Relation between penicillin concentration during pretreatment and subsequent
inhibition of nitratase adaptation in organism 1433
     Cells incubated for 2 hr at 37° C in salt solution containing $M/100$ NH$_4$Cl and $M/100$
glucose with penicillin concentrations as abscissae   Cells centrifuged down, washed once
with salt solution and then incubated for 90 min in salt solution containing $M/100$ NaNO$_3$,
$M/100$ glucose   Cells centrifuged down, washed in water and nitratase activity deter
mined   Ordinate = nitratase activity of penicillin pretreated cells expressed as per
cent activity of cells treated by same procedure in absence of penicillin

concentrations of penicillin , 50 per cent inhibition is obtained by pretreatment
with c 500 units penicillin per ml   The curve relating inhibition to penicillin
concentration shows an apparent discontinuity at the 60 per cent inhibition
level, and it was observed that cells which had been treated with concentrations
of penicillin greater than 600 to 700 units per ml showed a tendency to aggregate

It is possible that the greater inhibition obtained with higher penicillin levels was due to a secondary effect of the cell aggregation

The organism 143.3 possesses penicillinase, so that it is not possible to determine the concentration of penicillin inhibiting growth in a nutrient medium When tested by the usual serial dilution method, using an inoculum of c 10⁶ cells in casein-digest medium, growth was checked by 500 units penicillin per ml for 24 hours but took place at 1000 units per ml in 48 hours

Effect of external N-source

Cells were pretreated with 500 units penicillin per ml and then allowed to adapt to nitrate in the presence of various external nitrogen sources such as ammonia, lysine, glutamic acid, or an amino-acid mixture represented by vitamin-
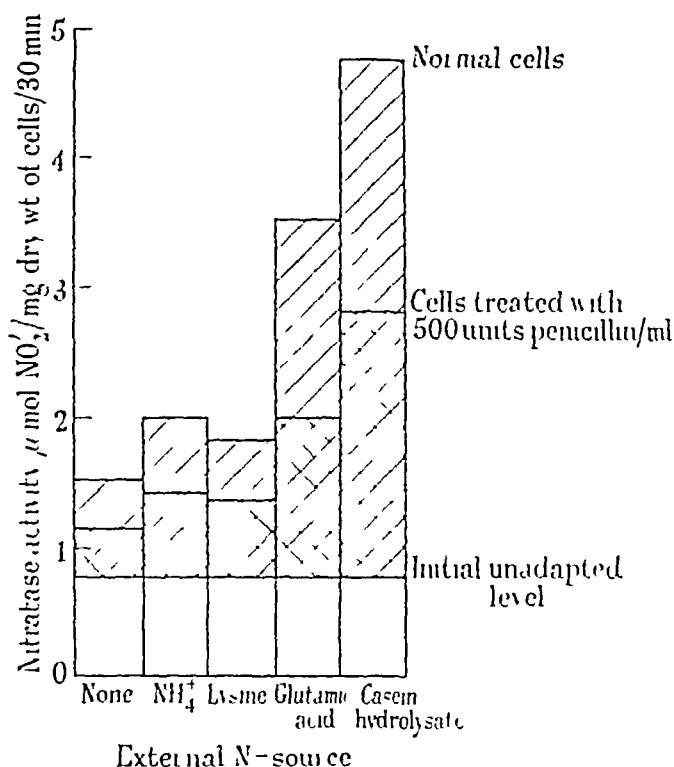

Fig 2 —Variation of nitratase adaptation of normal and penicillin treated cells with nature of external N source
        Cells incubated for 2 hr at 37° C in salt solution containing M/100 NH₄Cl and M/100 glucose with 500 units penicillin/ml (no penicillin in controls = normal cells)   After washing free from penicillin, cells incubated for 90 min at 37° C in salt solution containing M/100 NaNO₃, M/100 glucose and 1 per cent N source as above

free casein hydrolysate   In each case the adaptive increase was compared with that which had taken place in cells pretreated in the same way but in the absence of any penicillin   Fig 2 shows that, whatever the available N-source, the pretreatment with 500 units penicillin per ml has resulted in 50 per cent inhibition of the adaptive increase in nitratase activity   There is no evidence

that the adaptive increase stimulated by, say, lysine is affected differently from that stimulated by glutamic acid   The results suggest that it is the capacity to adapt which is affected by penicillin treatment rather than the assimilation of the available N-source

TABLE I —*Absence of Action of Penicillin on Glucose Fermentation*

Organism 1433 grown for 16 hr at 30° C on casein-broth-agar , harvested and washed once in salt solution   Fermentation studied in Warburg mano-meters containing 1 0 ml   M/5 phosphate buffer pH 7 0, 1 0 ml sus-pension of organism (= 5 mg dry weight of cells), 0 5 ml   M/100 glucose, 0 5 ml water or penicillin solution as below , manometers filled with O_2-free N_2 , CO_2 absorbed by 0 25 ml 10 per cent NaOH in centre pot Fermentation activity expressed as $Q_{H_2} = \mu l$ H_2 evolved/hr /mg dry weight of cells

Cell preparation	Penicillin concentration (units/ml)	Q_{H_2}
(1) Washed cells after harvesting	0	11
	500	11
	1000	8 5
(2) Cells incubated for 2 hr at 37° C in salt solution con-taining M/100 N$_4$HCl and 0 01 M glucose, washed and made up into suspension as (1)	—	31
(3) Incubated as in (2) but in presence 500 units peni-cillin/ml	—	40
(4) Incubated as in (2) but in presence 1000 units peni-llin/ml	–	39
(5) Cells incubated and prepared as in (4) but tested in presence M/20 adenosinetriphosphate	—	37

Effect of penicillin pretreatment on glucose fermentation

The energy for adaptive formation of nitratase, under the experimental con-ditions used, is derived from glucose fermentation   It is shown in the table that there is no significant difference between the rates of fermentation of cells whether pretreated with penicillin or not   Consequently the impairment of the adaptive capacity cannot be due to inhibition of the energy-supplying system

Reversal of the Penicillin Effect

Action of adenosine-triphosphate

The penicillin concentrations effective in these experiments are approximately the same as those which Gros and Macheboeuf (1948) find inhibit the breakdown of adenosinetriphosphate (ATP) and other nucleotides by *Cl sporogenes*   It seemed possible that, if the adaptive capacity were affected by an energy-trans-ferring mechanism, then nucleotide metabolism might be involved, and that modification of the penicillin effect could be obtained by adding ATP or related compounds to the cells during adaptation   Cells were treated with 500 units penicillin per ml and then rate of adaptive increase determined as before, and also in the presence of concentrations of ATP ranging from 1 to 10 mg Ba salt

per ml   The highei concentiations of ATP weie found to abolish the inhibitory action of penicillin   Fig 3 shows the effect of ATP concentration on the adaptation of both noimal and penicillin-treated cells, in the normal cells low concentiations of ATP increase the iate of adaptation, but high concentiations aic slightly inhibitoiy, in the penicillin-tieated cells low concentrations of ATP inciease the inhibition, whereas the inhibition is ieveised and completely
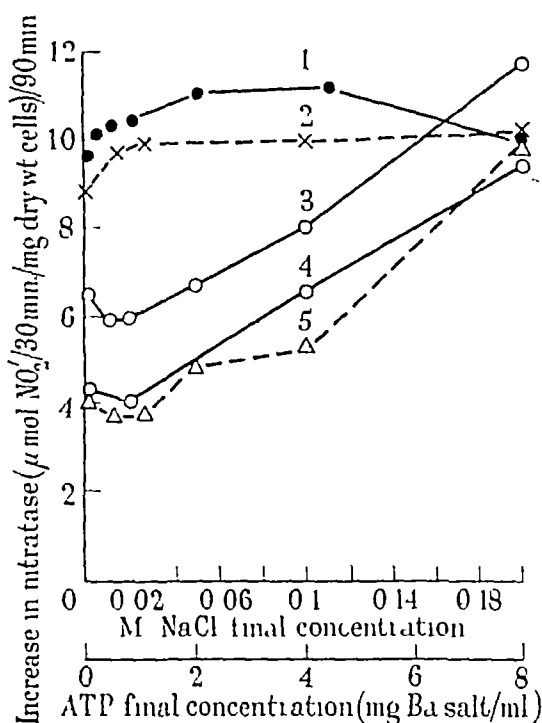

Fig 3 —Effect of adenosinotriphosphate preparation and of salt in removing penicillin inhibition of nitratase formation

Cells incubated for 2 hr at 37° C in salt solution with NH_4Cl and glucose and penicillin as before (see Fig 2)   After washing in salt solution, cells incubated for 90 min at 37° in salt solution containing м/100 $NaNO_3$, м/100 glucose, 1 per cent casein hydrolysate, and either ATP solution or NaCl to the final concentrations indicated   Cells then washed in water and increase in nitratase activity determined

Curve 1 Normal cells in presence of ATP preparation
   „   2 Normal cells in presence of NaCl
   „   3 Cells pretreated with 500 units penicillin per ml and adapted in presence of ATP preparation
   „   4 Cells pretreated with 700 units penicillin per ml and adapted in presence of ATP preparation.
   „   5 Cells pretreated with 700 units penicillin per ml and adapted in presence of NaCl

abolished by higher concentrations   The amount of ATP necessary to abolish the inhibition increases with the degree of inhibition, i e with the concentration of penicillin used for pretreatment   It was immediately obvious that the concentrations of ATP which are fully effective in abolishing the penicillin effect are very high, representing a weight of ATP many times greater than the weight of organism present   It seemed improbable that the effective agent in the ATP preparation was ATP itself   Preparations of adenylic acid, adenine, adenosine, ribose, etc were completely inactive   When the ATP preparation was

subjected to 10 min hydrolysis in N/1 HCl, and neutralized before test, it was found that its effectiveness in abolishing the penicillin effect was increased This suggested that the anti-penicillin action resided in the salt content of the preparation

Action of salts

Fig 3 shows also the effect of adding NaCl to the reaction mixture during adaptation   The action of the ATP preparations in abolishing the penicillin effect can be reproduced in all respects by suitable concentrations of NaCl   The ATP solution used contained a high concentration of salt, and in Fig 3 the ATP ordinates are made to correspond with the NaCl ordinates for like salt concentration, the action of the ATP preparation is fully explained by its salt content

The action of NaCl can be reproduced by Na_2SO_4 or KCl, similar actions being produced by solutions of similar ionic strength

Conditions for Reversal of Penicillin Action by Salt

The experiments described so far were all carried out with cells which were first treated with penicillin and then allowed to adapt in the absence of penicillin Salt was added during the adaptation process   Fig 4 shows that—

(1) The presence of salt during the treatment with penicillin does not prevent subsequent inhibition of adaptation

(2) After pretreatment with penicillin, salt abolishes the inhibition if present during the adaptation in the absence of penicillin

(3) If the cells are first treated with penicillin, then with salt, then washed and allowed to adapt in the absence of either salt or penicillin, the inhibitory action of penicillin is largely abolished

(4) If the cells are treated with penicillin, then allowed to adapt in the absence of penicillin or salt, and the adapted cells then treated with salt, the salt has no action in removing the inhibitory effect due to penicillin after adaptation has taken place

Consequently the antagonistic action of salt is shown only when the salt is added after the removal of the penicillin, and prior to or during the adaptation process

It was noted above that treatment of the cells with high concentrations of penicillin often resulted in aggregation of the cells during later stages of the experiment   This was particularly noticeable when penicillin-treated cells were incubated in the presence of casein hydrolysate (for the adaptation process) It was always found that the tendency to aggregate was lost whenever treatment with salt abolished the penicillin effect on adaptation

Experiments with Staphylococcus aureus

Assimilation of glutamic acid

Gale and Taylor (1947) found that if *Staph aureus* were grown in a medium to which penicillin was added 90 min before harvesting, the cells lost the ability to assimilate glutamic acid   It seemed possible that this effect might also be affected by salt   Consequently, *Staph aureus* was grown in the presence of

1 unit penicillin per ml under conditions identical with those previously described by Gale and Taylor (1947), and the ability of the cells to assimilate glutamic acid was tested with and without treatment with M/5 NaCl   Penicillin treatment completely blocked the passage of glutamic acid into the cells and this effect was not reversed by treatment with salt
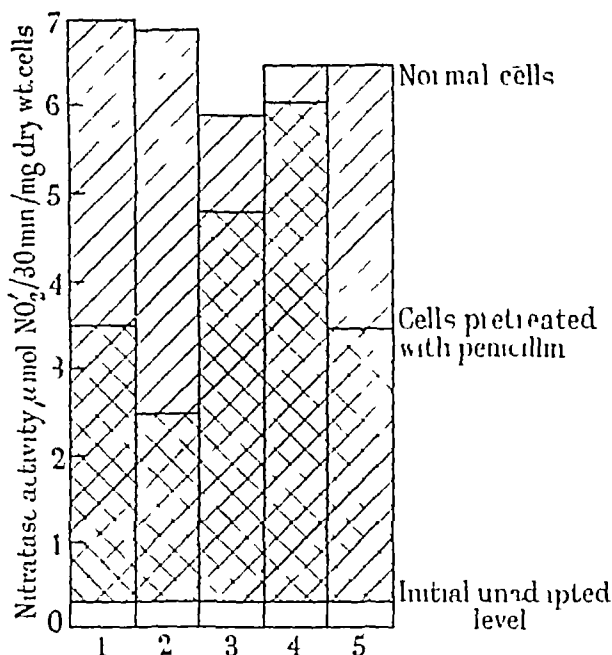

FIG 4 —Effect of salt on penicillin inhibition of nitratase formation
        Cells pretreated with penicillin and then allowed to adapt in the presence of nitrate, glucose and casein hydrolysate
        1   Cells pretreated with 700 units penicillin per ml   Adaptation process as usual
        2   Cells pretreated with 700 units penicillin per ml   in presence of M/5 NaCl , adaptation process as usual
        3   Cells pretreated with penicillin , washed , incubated for 30 min   in M/5 NaCl , washed , adaptation process as usual
        4   Cells pretreated with penicillin , washed , adaptation process in presence of M/5 NaCl
        5   Cells pretreated with penicillin , washed , adaptation process as usual , washed , incubated 30 min with M/5 NaCl before testing
    Controls (= normal cells) treated in parallel fashion in all cases in absence of penicillin during first stage

Nitratase adaptation

Staph aureus cells possess nitratase, and will undergo adaptation in a manner similar to that described for the organism " 1433 "   The adaptation is stimulated by the addition of single amino-acids as N-source   Cells were grown in penicillin (1 0 unit per ml) until their ability to assimilate glutamic acid was lost as above It was found that glutamic acid would still stimulate adaptive nitratase formation

DISCUSSION

        No explanation of the effects described in this paper can be put forward at the present time   During the course of the work it seemed clear that there

was some connection between the tendency of the cell suspensions to aggregate and an inhibition of the rate of adaptation   However, inhibitory effects were noted after treatment with penicillin in concentrations (100 units per ml and less) which did not give rise to later aggregation of the cells   The suspensions were examined under phase-contrast lenses, and, except where obvious aggregates were found after treatment with high concentrations of penicillin, the cells were motile and of the same appearance under all the conditions described in this paper   It seems probable that the treatment with penicillin produces some alteration in the surface of the cell, which is reversed by salt solution, and which is reflected in a slowing of the rate of adaptation to nitrate   Such effects might be expected if nitratase were an enzyme residing in or near the surface of the bacterial cell   There is no definite evidence that this is the case, although if the cells are disintegrated by mechanical agitation, nitratase activity is always associated with the solid particles (Pollock, private communication)   The fact that penicillin does not differentiate between ammonia and the various amino-acids as N-sources for adaptive protein formation might also be explicable if the enzyme were a surface one   The experiments carried out with *Staph aureus*, in which it was found that glutamic acid stimulates nitratase formation although its assimilation has been blocked by penicillin, would also be explicable if the site of nitratase synthesis were situated in the cell-surface at a level outside that of the osmotic barrier which normally restricts the entry of glutamic acid into the cell

It is clear that these effects deal with problems of enzyme location and of the nature of the bacterial surface, and explanations must await further developments in these fields

SUMMARY

(1) Incubation of cells of the coliform organism " 1433 " with high concentrations of penicillin results, after removal of the penicillin, in a decrease in their rate of adaptation to nitrate

(2) This effect of penicillin can be removed if the cells are incubated in the presence of high concentrations of salt prior to, or during, the adaptation process The presence of salt during the initial treatment with penicillin does not remove the effect   Treatment of the cells after adaptation has occurred does not restore the full nitratase activity

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SURVEY OF PAPERS

SVEDMYR finds that under suitable conditions a visible precipitate occurs immediately when a mixture is made of the haemagglutination inhibitor in allantoic fluid and influenza virus that retains its capacity of rendering inhibitor particles non-inhibitive The precipitate then gradually dissolves and free virus haemagglutinins reappear This precipitation-dissolution reaction reflects the course of the modifying effect of normal allantoic fluid on influenza virus haemagglutination (p 237)

SVEDMYR, on repeating the experiments described in the previous paper with influenza virus rendered incapable of destroying inhibitor particles, finds that precipitation occurs but the precipitate does not dissolve (p 248)

SVEDMYR finds that treatment of the inhibitor with virus or periodate destroys its capacity to form a precipitate with virus as described in his two preceding papers, whereas proteolytic enzymes have no such action (p 254)

KORPASSY AND KOVACS have shown that the prolonged subcutaneous administration of tannic acid to rats produces a diffuse nodular fibrosis of the liver Examination of the livers of animals killed at intervals during the experiment showed a progressive transformation of the architecture of the liver, the final picture being indistinguishable from human portal cirrhosis of the Laennec type (p 266)

DEKANSKI has modified the kaolin-adsorption method for concentrating normal urinary gonadotrophins, and has obtained relatively non-toxic concentrates suitable for further purification, isolation and standardization of the hormones (p 272)

HUMPHREY AND PAGEL have studied the reactions to injections of heated killed streptococci in the skin of normal subjects and of persons with rheumatic affections and subacute bacterial endocarditis They find no clear relationship between the intensity of the reaction during the first three days and the histological picture after ten to eighteen days (p 282)

HOLT has studied the production of antitoxin in guinea-pigs injected subcutaneously with 1 Lf of purified toxoid adsorbed on different quantities of aluminium phosphate carrier He finds that the enhanced potency of adsorbed toxoid is not due to the second response phenomenon or delayed release, but to a more efficient distribution of the toxoid among the antitoxin producing cells by the polymorphonuclear leucocytes (p 289)

HALE finds that the growth characteristics of a small colony variant of staphylococcus, isolated by cultivation on media containing gentian violet, depend on an impairment of uracil synthesis (p 297)

VAN HEYNINGEN describes the construction and operation of a laboratory circulating evaporator capable of evaporating water at the rate of 12–14 l per hour at 34–36° C and 20–30 mm Hg (p 302)

NEWTON describes four antibiotics produced by a strain of *B subtilis* Of these bacilipin A and bacilipin B are active mainly against *Myco phlei*, and bacilysin is active against *Staph aureus*, *C xerosis*, *Myco phlei* and *Bact coli* (p 306)

MILES finds that when diphtheria toxin is injected intradermally into a guinea pig it is absorbed immediately to the skin tissues A concentration gradient declining outwards is thus established so that the diameter of the lesion at 24 hours is proportional to the logarithm of the dose of toxin

injected   There is a certain degree of combination of toxin and tissue that can be reversed by antitoxin, so that the moment at which circulating antitoxin ceases to be effective is dependent on the rate of its passage across capillary endothelium and the rate of fixation of toxin to the tissues (p 319)

HUMPHREY finds that the level of antistreptolysin " S " in serum is not specifically correlated with streptococcal infection, and suggests that antistreptolysin "S" is not normally an antibody in the accepted sense of the word (p 345)

FISHER has studied the effects of diluents on prothrombin tests and finds that distilled water, 0 9 per cent sodium chloride solution and protein-free filtrate of normal serum give uniformly consistent results (p 352)

GALE finds that the rate of adaptation to nitrate of a coliform organism is decreased after incu bation with high concentrations of penicillin   This effect can be removed by treatment with high concentrations of salt (p 256)

THE NATURE OF ANTISTREPTOLYSIN S IN THE SERA OF MAN AND OF OTHER SPECIES   THE LIPOPROTEIN PROPERTIES OF ANTISTREPTOLYSIN S

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EVIDENCE has been presented elswhere (Humphrey, 1949) to suggest that antistreptolysin S does not behave as a true antibody, and that inhibition of streptolysin S is a non-specific property of the serum of numerous species Because of the interesting properties of streptolysin S, which in serum broth cultures appears to be a lipoprotein and in nucleic acid broth cultures to be a nucleoprotein (Herbert and Todd, 1944, Bernheimer and Rodbart, 1948), and because such non-specific inhibitors may play a part in natural resistance to disease, it seemed desirable to study further the nature of antistreptolysin S activity

A possible clue appeared to lie in the observation of Hewitt and Todd (1939) that streptolysin S is powerfully inhibited by lecithin in the absence of protein, particularly since the levels of serum lipoids have been found to fall during acute disease and to rise to normal or supra-normal values during convalescence (Peters and Van Slyke, 1946), in a rather similar manner to the antistreptolysin S titres An investigation of the effect of gentle extraction of the sera with lipoid solvents showed that such treatment did in fact remove much of the antistreptolysin S activity, but that the question was complicated by marked differences in behaviour between species and even between the sera of individuals within the same species Further work on the behaviour of antistreptolysin S after proteolytic digestion of the serum, and of its distribution among serum fractions, has thrown some light upon its nature, although this is far from being completely revealed

MATERIALS AND METHODS

Streptolysin S was obtained from a strain of streptococcus (Group A, Type 11) which was known to produce streptolysin S, but not streptolysin O   Two preparations were used, as previously described, one being a horse serum extract of streptococci grown in serum broth, and the other a filtrate of a yeast nucleic acid broth culture   Sera were stored frozen at − 20° C   Antistreptolysin estimations were performed as described by Todd (1938) with slight modifications In the case of streptolysin S the haemolysin was standardized against a human serum to which had been assigned an arbitrary value of 10 units per ml

* On the external staff of the Medical Research Council

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Lipoid extractions

Lipoid extractions were made with ether below − 25° C as described by McFarlane (1948) or with alcohol-ether at − 10° C as decribed by Hartley (1925) Three or four extractions were made with ether unless stated otherwise All solvents were of Analytical Reagent grade and were free from peroxides and all but traces of aldehyde

Lipoid phosphorus

Lipoid phosphorus estimations were made by a modification of the method of Youngburg (Hawk Oser and Summerson, 1947)

Cholesterol estimations

Cholesterol estimations were performed by the digitonide precipitation method of Schoenheimer and Sperry as modified by Hawk, *et al* (1947)

Total carbohydrates

Total carbohydrate estimations were made by the orcinol method of Sorensen and Haugaard (1933) in terms of glucose

EXPERIMENTAL

Inhibition of streptolysin S by lecithin, cholesterol and cholesterol esters

Preliminary experiments were performed to test quantitatively the inhibition of streptolysin S by lecithin and other lipoids Antistreptolysin S estimations were made in the usual way, and it was found that, in the absence of serum, ovo-lecithin was powerfully inhibitory to both preparations of streptolysin S A dispersion of highly purified lecithin at a concentration of 0 018 per cent in 0 9 per cent NaCl solution corresponded to 1 unit per ml antistreptolysin S When the streptolysin was diluted with normal rabbit serum however instead of buffer the inhibitory effect of lecithin was reduced by more than 9/10ths, and it seems unlikely that lecithin, in the concentrations found in normal serum could account for more than 0 5–1 unit antistreptolysin S per ml

One sample of cholesterol, of which a very fine suspension was obtained, inhibited streptolysin S comparably with ovo-lecithin This result was not in agreement with the findings of Hewitt and Todd (1939) and the experiments were repeated with recrystallized cholesterol It was not found possible to obtain so fine a dispersion of the recrystallized cholesterol as of the first preparation, but with it no significant inhibition was obtained Cholesterol esters (from wool fat) likewise caused negligible inhibition

The effect of neutral fat was tested by measuring the antistreptolysin S of the serum of an individual taken while fasting and after a fat meal Both the clear and lipaemic sera had the same antistreptolysin S titres

The effects of ether and alcohol-ether extraction

A number of sera both from normal and sick people and from other animals were extracted with ether below − 25° C or with alcohol-ether at − 10° C Treatment with ether removed nearly all free cholesterol, most of the ester cholesterol and a variable fraction of the phospholipids Alcohol-ether treatment removed nearly all detectable lipoids The antistreptolysin S content of the sera were measured before and after extraction and the results are given in

Table 1 In order to ensure that the treatment was not so drastic as to destroy other known antibodies, these were also estimated in a number of cases by conventional methods The results are summarized in Table II

TABLE I —*The Effect of Ether Extraction Upon the Antistreptolysin S Titres of Various Sera*

(Ranges are given in parenthesis)

Species	Number of sera	Mean antistreptolysin S units/ml		
		Before extraction	After extraction	Removed
Human	10	8 9 (6–12 5)	3 6 (1 5–5 6)	60%
Horse	6	16 5 (10–27)	3 5 (2 4–6 4)	79%
*Horse (anti-*welchii*)	6	5 5 (4 8–7 2)	2 5 (2 2–2 8)	55%
Rabbit	2	5	1 3	74%
Rat	2	55	6	89%
Ox	1	23	2 9	87%
Sheep	1	12	3 1	74%
Plaice	1 pool	48	23	52%
Guinea-pig	3 pools	46	43	6%

* Sera preserved with 0 3 per cent tricresol

Effect of Alcohol-ether Extraction

Human	4	8 7 (6 5–11)	3 7 (3 1–5 4)	58%
Horse	2	18 5	4 3	77%
Guinea-pig	1 pool	36	17	53%

TABLE II —*The Effect of Ether Extraction Upon the Titre of Various Antibodies in Sera*

(Ranges are given in parenthesis)

Species	Number of sera	Nature of antibody	Mean value		Removed
			Before	After	
Human	9	Antistreptolysin O units/ml	303 (28–1200)	260 (21–1000)	14%
Horse	1	Ditto	100	100	0%
Horse*	1	Diphtheria antitoxin Units/ml	420	390	7%
Horse+	5	Cl welchii β-antitoxin Units/ml	1300 (900–1800)	1250 (950–1800)	4%
Human	4	Iso-agglutinin titre	1 46	1 39	15%

The Effect of Alcohol Ether Extraction

Human	5	Antistreptolysin O Units/ml	872 (31–1200)	523 (20–810)	39%
Horse*	1	Diphtheria antitoxin . Units/ml	420	340	19%

* Estimations by Dr C L Oakley, Wellcome Physiological Research Laboratories, Beckenham

From Tables I and II it is clear that whereas lipoid extraction has a relatively minor effect upon a variety of known antibodies, it causes a major reduction in the antistreptolysin S titres of all the sera examined (human, horse, rabbit, rat, ox) except for guinea-pig, and possibly plaice sera, in which considerable anti-streptolysin S activity remained even after alcohol-ether treatment

It was of interest to examine the ether extracts for antistreptolysin S activity These were accordingly either recombined with the extracted sera or added to equivalent volumes of 0 9 per cent NaCl, and the ether was removed *in vacuo* over H_2SO_4 Both the saline and the serum preparations were very much more opalescent than the original sera, and a layer of fat formed on the surface after standing for some hours   Thus it was evident that the original lipoid complexes were not wholly restored

Representative results are given in Table III in which the behaviours of anti-streptolysin S and antistreptolysin O are contrasted   It will be observed that in each case the antistreptolysin S activity of the extracted lipoid only partly accounted for the difference between original and extracted sera, and that the reconstituted serum behaved with respect to antistreptolysin S as a simple mixture of the extracted serum and the lipoids   In the case of antistreptolysin O, however, the lipoid extracts had activities far greater than those of the original sera, yet the reconstituted sera were equivalent to the original sera   This rather puzzling observation is explained by the observation (Hewitt and Todd, 1939)) that *free* cholesterol is a very powerful inhibitor of streptolysin O   Thus lipoid extraction, while leaving the true antibody unchanged, frees cholesterol from the inactive protein complexes in which it occurs in serum   It is clear, however, that such complexes must be formed anew when the extracts are recombined, although this is not the case with the complexes concerned with antistreptolysin S activity

TABLE III —*Antistreptolysin S and O Contents of Sera Before and After Extraction with Ether, Compared with Titres of the Extracted Lipids Added to Saline and to the Ether Extracted Serum*

		Original serum	Ether extracted serum	Extracted lipoids	Reconstituted serum
Human A	A S S	10	4	1 5	5 8
	A S O	28	28	450	53
Human B	A S S	6 2	4	$<<1$	4 7
	A S O	1200	1000	2000	1200
Horse (anti-scarlatina)	A S S	13	2 5	<1	2 6
	A S O	100	100	900	100

The effects of proteolytic digestion

In order to disrupt the protein moiety of the lipoid complexes in serum use was made of proteolytic digestion   Of the available enzymes pepsin seemed suitable since it would be inactive at pH 8 0, even in the presence of reducing agents and since serum contains no natural inhibitor for it   Sera were treated with pepsin (B D H) at pH 3 and at 37° C until approximately 20 per cent of the protein nitrogen was no longer precipitable by tungstic acid   The sera were adjusted to pH 7, and the antistreptolysin S and antistreptolysin O contents were

measured before and after extraction with ether   Control samples were incubated under similar conditions without pepsin

Through the kindness of Dr C L Oakley there were available sera from horses immunized against *Cl welchii* which contained considerable amounts of β-antitoxin, and which had been subjected to pepsin treatment so brief as to leave the antitoxin titres substantially unchanged (Harms, 1948), The anti-streptolysin S and β-antitoxin of these sera were estimated before and after extraction with ether

The results are summarized in Table IV From this it will be seen that pro-longed incubation with pepsin, actually tended to increase the antistreptolysin S values of the sera, although antistreptolysin O was largely destroyed Further-more, after such prolonged treatment almost all the antistreptolysin activity could be extracted by ether From the control experiments without pepsin it appears that most of these effects were obtained by incubation at pH 3 alone

TABLE IV —*Effect of Pepsin Treatment upon Antistreptolysin S, Antistreptolysin O, and Anti-β-toxin Contents of Sera*

(Mean values are given as percentages of those of the original sera)

Treatment	Type of serum	Number	Antistreptolysin S		Antistreptolysin O	
			Before ether	After ether	Before ether	After ether
Prolonged with pepsin	Human	3	190%	9%	7%	Not done
	Horse	2	100%	6%	Not done	,,
Prolonged with-out pepsin	Human	2	100%	6%	1%	,,
	Horse	2	78%	6%	Not done	,,
					Anti β toxin	
Brief with pepsin	Horse	5	135%	62%	77%	71%

Brief incubation with pepsin also caused a rise in antistreptolysin S, but in this case there was no increase in extractability with ether Presumably under such mild conditions (in which the β-antitoxin was largely unchanged) the lipo-protein complexes were only slightly disrupted

The correlation between serum lipoids and antistreptolysin S titres

The experiments described above suggested that lipoid-protein complexes were responsible for the greater part of the antistreptolysin S activity of sera, with the possible exceptions of guinea-pig and plaice sera In order to test whether any direct correlation could be found between the presence of particular lipoid constituents and antistreptolysin S activity, the lipoid phosphorus and free and total cholesterol content of a number of sera from different species were estimated, both before and after extraction of lipoids From Table V it is apparent that there was no correlation between antistreptolysin S titres and any of the serum constituents measured It is particularly notable that the alcohol-ether extracted sera contained only very small amounts of phospholipid or cholesterol, and yet had considerable residual antistreptolysin S

TABLE V —*Correlation Between Antistreptolysin S and Serum Lipoid Content*

Species	No	Antistreptolysin S (units/ml) Range	Antistreptolysin S (units/ml) Mean	Mean lipoid P (mg /100 ml)	Mean free cholesterol (mg /100 ml)	Mean total cholesterol (mg /100 ml)
(1) *Untreated sera*						
Human	6	9–13	10	8 4	65	161
	8	7–9	7 8	6 3	37	138
,,	12	2–7	5 5	6 8	42	140
Horse	2	—	18 5	4 3	20	89
Rat	2 pools	—	55	1 4	0	31
Guinea pig	1 pool	—	35	1 72	Not done	37
Sheep	1	—	12	3 2	,,	53
Plaice	1 pool	—	50	9 5	,,	109
Dog fish	,,	—	5	3 1	,,	104
Spider crab (whole blood)	,,	—	1 2	0 24	,,	<10
(2) *Ether extracted sera*						
Human	4	3 9–5 6	4 5	7 2	2	18
Horse	2	—	6	2 5	11	21
Rat	2 pools	—	6	1 3	0	5
Sheep	1	—	3 1	1 9	Not done	<10
(3) *Alcohol ether extracted sera*						
Human	3	—	3 7	0 5	0	7
Horse	2	—	4 4	0 25	0	0
Guinea pig	1 pool	—	₁7	0	0	0

The antistreptolysin S content of serum fractions

Effect of dialysis —The distribution of antistreptolysin S activity in serum between the euglobulin fraction (precipitated by prolonged dialysis in the cold against distilled water saturated with CO_2) and the remainder was studied in several sera (Table VI) Somewhat surprisingly it was found that the two

TABLE VI —*Distribution of Antistreptolysin S Between Euglobulin Fraction and Residue in Various Sera*

Serum	Original titre (units/ml)	Per cent original activity in Euglobulin	Per cent original activity in Remainder	Per cent original activity in Recovery
Human	12	7	81	98
Ox	23	130	108	238
Guinea-pig	32	146	148	264
Horse 7629	27	80	30	110
(after ether extraction)	4	58	19	77
Horse 7637	10	45	100	145
,, (after ether extraction)	3 3	70	30	100

fractions of ox guinea-pig and horse sera taken together had more antistreptolysin S activity than the original serum Furthermore, the two horse sera differed sharply from one another—that with a high antistreptolysin S having relatively a much larger activity in the euglobulin than the other, while the activity of the remaining fractions was about equal After ether extraction the two horse

seia behaved similaily, and then euglobulin fiactions contained almost 3/4 of the residual antistieptolysin S

These experiments demonstrated that the sepaiate antistieptolysin S activities of seium components weie not necessaiily equal to the activity of the original serum, and that not only weie theie diffeiences between species but also between individuals within a species   It was neveitheless decided to attempt to follow the behaviour of antistieptolysin S in human serum fractionated by ammonium sulphate and by electrophoresis

Ammonium sulphate fiactionation —Two normal human seia, before and aftei extraction with ethei, were fractionated with ammonium sulphate solution (buffered at pH 6 7) in the cold   The fiactions were freed from ammonium sulphate, by dialysis in the cold fiist against distilled water and then against 0 9 per cent NaCl   The antistreptolysin S and O, and the nitrogen, total cholesterol, lipoid phosphoius, and total carbohydrate content of each fraction were then estimated   Table VII shows the distribution of antistreptolysin S and antistreptolysin O between the fractions   The percentages are given in terms of the original serum activity, and the differences between the two sera are largely due to poorer recovery of activity in serum B than serum A

TABLE VII —*Peicentages of Original Antistreptolysin S and O Activity Recoveied in Different Fiactions of Human Serum*

	Per cent saturation with ammonium sulphate							
	0–33		33–40		40–50		50–80	
Serum	S	O	S	O	S	O	S	O
Human A	7	77	9	14	16	9	59	0
Human A (ethei extd)	7	64	7	9	7	2	7	0
Human B	5	53	6	31	8	16	58	0
Human B (ether extd)	3	30	4	15	4	8	4	0

Electrophoietic Fractions

	γ glob		β- + γ glob		α glob + alb		Albumen	
Antistreptolysin	S	O	S	O	S	O	S	O
Human C	(?5)	58	(?12)	42	16	0	67	0

It is clear, however, that (in contrast to antistreptolysin O) the antistreptolysin S activity is mainly in the albumen containing fractions, and that it is from these that the activity is removed by treatment with ether

No quantitative correlation was found (Table VIII) between the antistreptolysin S and the total cholesterol, the lipoid phosphorus, the protein nitrogen oi the total carbohydrate in the fractions of the unextracted sera, and although after ether extraction there was apparently a fair correlation between caibohydrate content and antistieptolysin S in serum A, there was more variation in serum B

In contrast to the results obtained with the human sera, similai fractionation of pooled guinea-pig serum, before and after extraction with alcohol-ethei, showed that the antistreptolysin S activity was initially high both in the albumen and globulin fractions, and that after extraction the activity of the globulin fraction was diminished more than that of the albumen

TABLE VIII —*Correlation of Antistreptolysin S and Serum Constituents in Fractions of Serum A*

| | Antistreptolysin S in units per mg of | | | |
	Nitrogen	Cholesterol	Lipoid P	Total carbohydrate
Before extraction				
0–33% Am Sulph	0 33	8 1	136	5 4
33–40% ,, ,,	0 78	6 8	238	8 9
40–50% ,, ,,	1 59	9 1	200	16 5
50–80% ,, ,,	1 06	5 2	107	22 7
After ether extraction				
0–33% Am Sulph	0 29	17	84	5 0
33–40% ,, ,,	0 37	9	70	6 5
40–50% ,, ,,	0 79	38	64	7 0
50–80% ,, ,,	0 14	10	37	6 2

Electrophoretic fractionation—Through the kindness of Dr C L Oakley an electrophoretic separation was performed at the Wellcome Research Laboratories on a sample of normal human serum   The separation was carried out at pH 8 0 in phosphate buffer, $\mu = 0\ 2$ Albumen, albumen $+$ σ-globulin, $\beta-+\gamma$-globulin and γ-globulin fractions were examined for antistreptolysin S and O

The results were in agreement with those found with the ammonium sulphate fractions, except in one respect   It was found impossible to measure the antistreptolysin S activity of the fractions containing γ-globulin when yeast nucleic acid broth streptolysin was used, irregular results with apparently very high titres being obtained despite several repetitions of the assay   When serum streptolysin, which in other estimations behaves in the same way as nucleic broth streptolysin, was used for the assay end-points were obtained, but the apparent antistreptolysin S activities both of the γ-globulin and of the $\beta-+\gamma$-globulin fractions were still greater than the total activity of the original serum   Similar phenomenona, though less well marked, were observed when estimating antistreptolysin S on separated ox and guinea-pig euglobulin fractions, and it appears that the combination between γ-globulin and streptolysin S is very much firmer in the absence of other serum components

The effect of acid, alkali and heat

It has already been mentioned (Table IV) that incubation of serum at pH 3 for 48 hr caused only a small decrease, if any, in the antistreptolysin S content   Other samples of normal human serum—untreated, ether extracted and reconstituted after ether extraction—were kept at pH 9–10 for 3 days in the cold without significant change in antistreptolysin S   The same samples were heated at 65° C for 1 hour also without effect upon the antistreptolysin S

Heating of normal and ether extracted horse serum at 100° C for 5 minutes caused coagulation   The clear fluid which separated from the coagulum in both cases had considerable antistreptolysin S activity approximately equal to that of the unheated ether-extracted serum   This activity was shown to be associated with uncoagulated protein being reversibly precipitated by trichloracetic acid, and was not affected by further extraction with ether
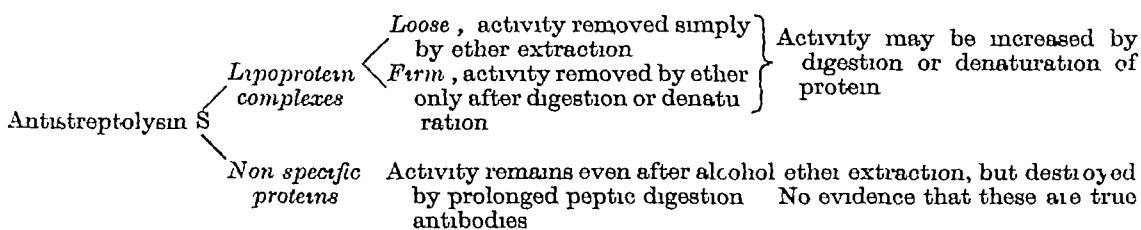
The effect of varying treatment with ether

It had been observed that successive extractions of serum with ether below − 25° C., up to four in number, all removed antistreptolysin S activity, though in diminishing amounts. It was of interest to note the effect of saturation of serum with ether, with gentle shaking, followed by removal of ether by evaporation on a pump so as to leave behind all the original serum components. When a horse and a human serum were thus treated at room temperature then antistreptolysin activities fell to 55 per cent and 71 per cent, and when treated below − 25° C to 26 per cent and 40 per cent respectively. Thus it is evident that the lipoprotein complexes concerned in antistreptolysin S activity are extremely labile to ether and are irreversibly broken simply by saturating at room temperature.

DISCUSSION

The evidence presented above supports the suggestion made on serological grounds that antistreptolysin S is not an antibody in the accepted sense, and furthermore that its nature varies between species, and even between individuals. In human serum most of the antistreptolysin S activity is carried by a labile lipoprotein complex associated with the albumen fraction, but the globulin fractions also have some activity, most of which is retained after gentle treatment with fat solvents.

Mild denaturation or peptic digestion may increase the antistreptolysin S activity by loosening the lipoprotein complexes without destroying that part of the proteins which carries the residual antistreptolysin S activity after ether extraction. More extensive digestion of the proteins, however, destroys this residual antistreptolysin S. Schematically this may be represented as follows

Antistreptolysin S
— Lipoprotein complexes
  — *Loose*, activity removed simply by ether extraction
  — *Firm*, activity removed by ether only after digestion or denaturation
  ⎱ Activity may be increased by digestion or denaturation of protein
— *Non specific proteins* Activity remains even after alcohol ether extraction, but destroyed by prolonged peptic digestion. No evidence that these are true antibodies

The same scheme would also cover horse sera, with the proviso that in some sera a considerable amount of active lipoprotein complex may be found in the euglobulin fraction. Insufficient evidence was obtained for generalization about other species, but in guinea-pig serum, which behaved differently from other sera, lipoprotein complexes are either unimportant in this connection or else are so stable that they are unaffected by ether treatment.

Concerning the detailed nature of the inhibitors mainly negative evidence has emerged, for there was no apparent correlation between antistreptolysin S and any of the serum constituents examined. The methods of fractionation used were too crude to permit identification of the protein, or of the lipoprotein factors, which were included in the globulin fractions. Some information was gained by examination of the streptolysin S inhibition by human serum fractions prepared for another purpose by the cold ethanol fractionation method of Cohn and his colleagues (Edsall, 1947). Considerable inhibition was caused by the fractions containing the β_1 lipoprotein (Fraction III_0), the σ_1 lipoprotein (Fraction IV_1),

and by the γ-globulin containing Fraction (II + III$_w$)   The albumen fraction, however, presumably because of the alcohol treatment involved in fractionation, caused negligible inhibition   Fraction II and III (which contains the inhibitory III$_0$ and II + III$_w$) was notably less active than either of its constituent fractions demonstrating once again that inhibition of streptolysin S is a complex phenomenon, and that the affinity of a particular serum component may depend upon the presence or absence of others   Greater knowledge of streptolysin S itself is required before the exact mechanism of its inhibition can usefully be considered

It may be of interest to compare antistreptolysin S with some other inhibitors of enzymes or toxins known to occur in normal sera   Duthie and Lorenz (1949) have recently studied trypsin inhibitor, and an inhibitor of the proteases of certain non-pathogenic bacteria, and have reviewed earlier work on these substances Trypsin inhibitor is found mainly in the albumen fraction, although some activity was present in all fractions of serum examined   Unlike antistreptolysin S it is relatively unaffected by ether treatment, although much diminished by chloroform   The bacterial protease inhibitor was found to be a labile component of the globulin fraction, being present in varying amounts in different species Plasmin inhibitor, studied by MacFarlane and Pilling (1946) and others, appears to resemble trypsin inhibitor in its behaviour   Among inhibitors of haemolysins may be mentioned an inhibitor of θ-toxin of *Cl welchii* (private communication from Dr C L Oakley), and a non-specific inhibitor of streptolysin O found by Packalén (1948) in sera from cases of hepatitis   The latter appears to be a lipoprotein present in α- and β-globulin fractions

Normal sera also contain an inhibitor of hyaluronidase (McClean, 1942) and an inhibitor of the haemagglutinating and certain other properties of influenza virus which is found mainly in the β_1 globulins (McCrea, 1946 , Hirst, 1949)   In connection with this so-called " Francis inhibitor " it has been suggested by Smith and Westwood (1949) that such inhibitors may be biologically important in determining natural resistance to particular infectious agents   It is possible that the widespread occurrence of antistreptolysin S may account for the apparent unimportance of streptolysin S production in determining the virulence of streptococci

SUMMARY

(1) Treatment of sera with ether or alcohol-ether, in such a manner as to cause only minor changes in the titres of known antibodies, causes a major reduction in antistreptolysin S titre   This phenomenon was observed in the sera of man, horse, rabbit, rat, ox, sheep, but was less marked in guinea-pig and plaice sera

(2) From a study of the effects on antistreptolysin S of peptic digestion of sera and of their fractionation by various methods, it is concluded that in man and the horse antistreptolysin S activity is a complex phenomenon, mainly dependent upon labile lipoprotein components associated with the albumen fraction

(3) The antistreptolysin S activity of separated serum components may be greater than that of the original serum, presumably because some of the groups capable of linkage with streptolysin S are normally linked with one another

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EXPERIMENTAL BERYLLIUM POISONING

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THE work to be described in this paper was begun in January, 1947, to provide information on the toxicity of beryllium and its compounds   Hyslop, Palmes, Alford, Monaco and Fairhall (1943), in a long report, stated that no particular toxicity had been established for beryllium, and that whatever toxicity had been found with the salts of beryllium was due to the toxicity of the acid radicle and the hydrolysis of the salts   These writers had made an extensive review of the literature, but failed to emphasize the importance of earlier work by Siem (1886) and Comar (1935) which demonstrated the toxic properties of beryllium   When our preliminary experiments demonstrated beyond doubt that beryllium was a very toxic element, it became possible to assess the results of previous workers, and to explain many of the discrepancies and contradictions found in the literature   Hyslop *et al* (1943) include in their report a very complete bibliography, and no useful purpose will be served by providing a similar list in this paper   It should,

however, be emphasized that confusion has arisen because both soluble and insoluble compounds have been used, and because no serious attention has been given to the varying effects produced by different routes of administration

When given intravenously the soluble salts of beryllium have always shown themselves to be toxic in doses so small that neither the acidity of the solution nor the properties of the anion could be held responsible for the liver necrosis and death of the animal Our experiments have confirmed the earlier ones of Siem (1886) and Comar (1935), and in a recent paper Scott (1948) has described the pathological lesions produced by beryllium administered intravenously

Having established that beryllium was in fact toxic, we carried out further work in an attempt to find out more about the mode of action of beryllium, and the genesis of the very profound tissue reactions that it produced

METHODS AND MATERIALS

A method suitable for the microdetermination of beryllium in biological materials has been described elsewhere (Aldridge and Liddell, 1948) It is a long and difficult method, requiring considerable care and experience before reproducible results can be obtained For these reasons the number of determinations made had to be limited Further, the method cannot be used for determining beryllium in bone or faeces, low values obtained in control experiments with these materials were assumed to be due to the mechanical entrainment of beryllium compounds in the large amount of calcium sulphate deposited in the sulphuric acid digest

For some of the work a roughly quantitative spectrographic method was employed, and a few spectrographic determinations of beryllium in bone were also carried out for us at the Atomic Energy Research Establishment, Harwell

Standard histological methods were used for the examination of the tissues Much valuable information was obtained from the use of a special histochemical method for staining beryllium in fixed tissues The method will be described in full elsewhere

Rats—albino and hooded—were bred at the Animal Farm, Chemical Defence Experimental Establishment The greater bulk of the work was done with these animals, which were in good condition and free from intercurrent disease Mice and rabbits were obtained mainly from dealers The rabbits varied very considerably, and many were heavily infected with coccidia

Beryllium compounds used were prepared from metallic beryllium of at least 99 9 per cent purity Beryllium sulphate, oxyfluoride and lactate were used and, in addition ammonium beryllium oxalate and sodium beryllium tartrate and malate were prepared for certain experiments Beryllium lactate is a basic lactate whose composition varies with the conditions of preparation For this reason all samples were analysed for their beryllium content It is freely soluble in water, giving a clear solution of about pH 5 5 Ammonium beryllium oxalate is a double salt in which beryllium is ionized Beryllium also forms complex compounds with sodium tartrate and malate In these salts beryllium is contained within the anion and is not ionized The compounds are freely soluble, but the beryllium is not precipitated by phosphate They have been assigned the following formulae (Parsons, 1909)

$$\left[\begin{array}{cc} \text{C O O Na} & \text{Na O O C} \\ | & | \\ \text{C H O} & \text{O H C} \\ | \quad \diagdown \text{Be} \quad \text{Be} \diagup \quad | \\ \text{C H O} & \text{O H C} \\ | & | \\ \text{C O O} \quad \text{Be—O—Be} \quad \text{O O C} \end{array}\right],10H_2O \qquad \left[\begin{array}{cc} \text{C O O Na} & \text{Na O O C} \\ | & | \\ \text{C H}_2 & \text{H}_2\text{ C} \\ | & | \\ \text{C H O—Be—O—Be—O H C} \\ | & | \\ \text{C O O} \quad \text{Be—O—Be} \quad \text{O O C} \end{array}\right],7H_2O$$
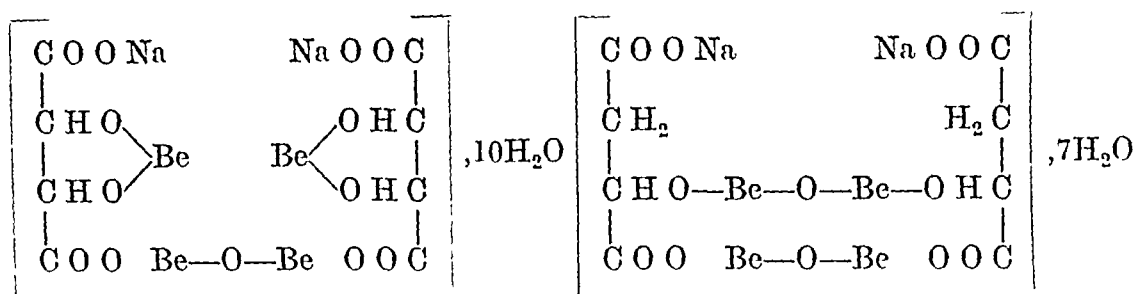
Every sample of beryllium lactate, tartrate and malate was analysed for beryllium

All the earlier work was carried out with beryllium lactate because solutions of this salt are only slightly acid and the anion can be considered completely non-toxic

RESULTS

Toxicity of Soluble Beryllium Compounds by Intravenous Injection

Solutions of beryllium lactate and sulphate were injected intravenously into rabbits, rats and mice When the dose of beryllium is equivalent to 0 5–1 mg per kg body weight, death of the animal usually follows 1 to 4 days later An average lethal dose of the same order was reported by Siem (1886) and Comar (1935) with cats, dogs and rabbits injected with beryllium tartrate, lactate and sulphate

The LD50 for beryllium lactate was calculated for mice (20 g) and rats (200 g) The figures are presented in Table I Young rats are somewhat more resistant than older animals

TABLE I —*The Mortality Rates and LD50 for Beryllium Lactate Injected Intravenously into Rats and Mice*

Mice Weight 20 g		Rats Weight 200 g	
Dose mg Be/kg	Deaths/total injected	Dose mg Be/kg	Deaths/total injected
0 25	7/20	0 3	2/20
0 5	13/20	0 6	25/40
0 75	19/20	0 9	18/20
LD50	0 34 mg /kg		0 53 mg /kg
Fiducial limits	0 23—0 42		0 44–0 60

Rabbits seemed slightly more sensitive in that a dose of 0 5 mg of beryllium per kg killed all of the 37 animals injected, but the high incidence of coccidial infection with attendant liver cirrhosis may have been partly responsible

There was no immediate reaction to the injection of an average lethal dose, but within 18 hours the animals were obviously ill, sitting quietly and refusing food This condition persisted until death In the case of rats and mice death took place unobtrusively, but in many rabbits a short series of violent convulsions immediately preceded death The rats, if they survived 3 days or longer, became jaundiced

At post mortem all animals showed a striking degree of liver damage, obvious

to naked eye inspection If death was early the liver was red, friable and haemorrhagic, but in later deaths the liver became yellow and soft   In rabbits there was a variable amount of haemorrhagic peritoneal exudate   The renal surfaces often had superficial haemorrhages, as did also the pleural surfaces of the lungs   These changes were only seen in the rabbits, and may have been partly the result of the terminal convulsions   One other noticeable feature was the delayed clotting time of the blood from animals dying after beryllium poisoning

There was very little variation in the general picture of beryllium poisoning seen among animals which were the subject of experiments spread over more than 2 years

The claim of Hyslop et al (1943) that the toxicity of beryllium compounds was connected with the properties of the anion or of the salt as a whole, rather than being a specific property of beryllium, seemed to be adequately discountenanced by these experiments with beryllium lactate   The toxicity of a number of beryllium salts was compared in an experiment with mice in which the animals each received 10 μg of beryllium in solutions of different salts   The results, presented in Table II, show that there is no marked difference in the toxicity of the different salts with the exception of sodium beryllium malate   It is possible that the explanation may be in the rate of breakdown of this salt to give a slow release of beryllium into the tissues

TABLE II —*The Comparative Toxicity of Different Salts of Beryllium*

Animals   Mice (20 g)
Number per group   30
Dose of beryllium injected   10 μg /mouse
Volume   0 1 ml
Route   Intravenous

Salt	Mortality per cent
Beryllium sulphate	77
Beryllium oxyfluoride	88
Beryllium lactate	66
NH$_4$ beryllium oxalate	67
Na beryllium tartrate	80
Na beryllium malate	13

The toxicity of beryllium was also compared to that of lithium, magnesium, calcium and aluminium injected intravenously into mice as solutions of their lactate   Whereas a dose of 20 μg beryllium killed every mouse, the molar equivalent of 40 μg beryllium as lithium, calcium or aluminium did not kill a single mouse   Magnesium at this level produced acute symptoms which were occasionally fatal within a few minutes, but the surviving animals recovered completely

The Fate of Beryllium after the Intravenous Injection of its Soluble Salts

The method of estimation devised by Aldridge and Liddell (1948) made it possible to determine the amount of beryllium down to 0 5 μg in all tissues except bones and faeces   A number of determinations were therefore made of the amount of beryllium held in the different tissues at varying times after the intravenous injection of beryllium salts

In a preliminary experiment a rabbit was injected with 7 mg of beryllium per kg as a solution of beryllium lactate   Spectrographic analysis of the blood showed that within 6 hours all but a trace of beryllium had left the circulation   The organs of this animal were analysed by the chemical method after death, 44 hours after the injection   The blood, heart, lungs, liver, spleen, kidneys, skeletal muscle, and brain were examined   About 30 per cent of the total beryllium injected was accounted for, and of this, 40 per cent was in the liver and 48 per cent in the spleen

A further series of experiments was carried out with mice   In each case the animal received 80 μg beryllium in 0 1 ml of a solution injected into the tail vein   Animals were killed at different times after injection and the organs analysed for beryllium   The results of several experiments are presented in Table III   After a dose of this size mice do not live as long as 48 hours, and so it was not possible to see whether beryllium concentrated in the spleen after 24 hours

TABLE III —*The Beryllium Content of Organs of Mice Killed at Different Times After the Intravenous Injection of 80 μg of Beryllium*

Organ	Time after injection of beryllium			
	5 minutes	1 hour	2 hours	24 hours
Liver	10 8 (3)	24 8 (8)	29 5 (6)	22 0 (7)
Spleen	0 (3)	2 8 (8)	2 0 (6)	2 1 (7)
Kidney	2 1 (3)	0 8 (8)	0 6 (6)	0 7 (7)
Lung	0 (3)	1 2 (7)	0 6 (6)	0 6 (6)
Gut	0 (3)	3 3 (8)	3 3 (6)	2 3 (7)

Percentage beryllium recovered

a Total	16	40	45	34
b In liver	13 5	30	31	27 5

Beryllium (μg)expressed as average of a number of experiments (in brackets)

In addition the total beryllium recovered is expressed as a percentage of the amount injected, and this never exceeds 50 per cent   The heart, brain, skin and skeletal muscles were examined, but never contained any measurable amounts of beryllium   The tail was also analysed to check any possible loss of beryllium round the vein at the time of the injection but only an occasional trace was recovered   In a later experiment 12 mice were each injected with 80 μg of beryllium into the tail vein   Four were killed 1/2, 6 and 24 hours after injection   The total beryllium in the skeleton of each mouse was determined spectrographically, and amounts varying from 2 1 to 8 5 μg per mouse were found   There was no evidence of any accumulation of beryllium in the skeleton within the first 24 hours of a single intravenous injection   The total beryllium in the skeleton never accounted for more than 10 per cent of the injected beryllium

The excretion of beryllium after the intravenous injection into rats was determined and did not exceed 10 per cent of the total injected, even in animals that lived for 72 hours

Although we have not found it possible to trace all the injected beryllium, it is noteworthy that of the amount that can be recovered, by far the greater part is in the liver   The figures for mice are given in Table III   The figures for rats

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are similar (Table IV) However, in early experiments with rats, in which a small weighed sample of the liver was taken, the results were extremely inconsistent It was then discovered that the concentration of beryllium in different parts of the liver varied very considerably, and so the content of the whole liver could not be calculated from a determination made on a small sample In Table IV the figures are given for the concentration of beryllium in the four main lobes of the livers of 7 rats The variations in concentration in a given animal are considerable, but there is no fixed ratio of the amounts of beryllium in the different lobes This suggests that there is no anatomical basis to explain these variations The total amount of beryllium found in the liver 20 hours or more after injection was about 30 per cent of the total injected

TABLE IV —*The Concentrations of Beryllium in the Separate Lobes of the Livers of Rats Injected with* 200 µg *Beryllium Intravenously and Killed at Intervals After*

Animal No		1	2	3	4	5	6	7
Time after injection		7 min	15 min	4 hr	20 hr	20 hr	20 hr	48 hr
Concentration of beryllium (µg /g liver)								
Lobe	Right anterior	1 7	6 6	10 0	8 2	8 4	10 3	6 2
	Left anterior	5 9	4 9	9 9	5 2	7 7	5 1	3 6
	Right posterior	3 9	5 0	15 5	16 5	4 3	3 3	3 3
	Left posterior	6 0	7 5	11 5	7 4	9 1	4 5	4 6
Total beryllium in liver (µg)		29	33	41	53	52	69	47
Percentage of injected beryllium		14 5	16 5	20 5	26 5	26	34 5	23 5

(Beryllium as µg /g of liver)

The distribution of beryllium in the liver of the rabbit was found to be more nearly equal in all parts The analysis of 5 samples taken from different lobes of a liver of a rabbit, dying 48 hours after the injection of 0 2 mg /kg , showed a variation of only 1 55 to 1 93 µg /g in the concentration of beryllium Again, about 40 per cent of the total beryllium injected could be recovered from the livers of rabbits 24 hours or more after the injection

The results of the tissue analyses for beryllium indicated that while some 40 per cent of injected beryllium could not be traced, the greater part of the remainder collected in the liver and remained there until the animal died Only a small percentage was excreted, and there was no evidence of any concentration in bone over the short period of these acute experiments

The Tissue Reactions to Beryllium

The tissue reactions to the intravenous injection of beryllium salts have been studied by a combination of the usual histological staining methods with a special histochemical method devised by Denz (1949) With the aid of the latter method it was possible to stain the tissues specifically for beryllium The method consists of forming a coloured compound with beryllium and the dyestuff " naphthocrome Green B " under conditions in which iron, aluminium and other elements normally present in the tissues do not react with the stain The validity of the results can be shown by a comparison of the intensity of the staining seen in different organs with their beryllium content estimated chemically, e g

	Liver	Spleen	Kidney	Lung	Heart
Concentration of beryllium (μg/g wet tissue)	29 5	2 0	0 6	0 6	0
Intensity of beryllium stain	+++	++	+	−	−

The histochemical method is not sufficiently sensitive to detect beryllium in the tissues after the injection of a single lethal dose, and as a rule histochemical tracing was carried out after the injection of 8 M L D

The distribution of beryllium after 8 M L D might be very different from the distribution after the injection of 1 M L D   However, it is just possible to detect beryllium in the tissues after 2 M L D, and the distribution is the same as after 8 M L D

The distribution of beryllium and the histological changes in the different tissues are described in detail below

Liver

Within a few minutes of the intravenous injection of beryllium lactate beryllium can be seen within the arteries and veins of the liver, and the parenchyma takes on a pale green tint   Within 10 minutes beryllium begins to collect in the sinusoids   By 30 minutes the diffuse staining has reached a maximum, and thereafter decreases slowly   But the staining of beryllium in the sinusoids increases in intensity up to 2 hours, and persists for 24–48 hours while pathological lesions are developing   A few hours after the injection of beryllium the liver presents a characteristic picture, with the pale diffuse green staining of the parenchyma speckled with the darker green of focal concentrations within the sinusoids   These focal concentrations of beryllium appear to consist of protein masses staining uniformly for beryllium and lying within the lumen of the sinusoids   They are found predominantly in the midzone of the lobule, though occasional aggregations are seen in the central and peripheral zones   There is some evidence of the phagocytosis of these beryllium protein masses by the Kupffer cells, but this does not account for the focal accumulations of beryllium

Pathological changes appear in the liver within three hours of the intravenous injection of a dose of 1 mg beryllium per kg   The normal pattern of the liver is replaced by a uniform field of swollen cells which have become hexagonal in outline as a result of common pressure   This generalized reaction disappears within 24 hours   Instead, the sinusoids of the midzonal regions now become dilated and many are grossly distended   Focal lesions in the liver cells become conspicuous   These focal lesions may be detected within 6 hours, and consist of small groups of necrotic parenchymatous cells   The cytoplasm of these cells becomes pale and vacuolated, the cell outlines are indistinct and the nuclei either hydropic or small, dark and pyknotic   Groups of damaged cells sometimes form rosettes around midzonal sinusoids

These changes are taking place while the focal concentration of the beryllium in the sinusoids is increasing, and around these areas focal necrosis develops   Within 24 hours the general reaction of the liver has subsided, and some time before this the general staining of the parenchyma with beryllium has faded   Meanwhile, around the tiny focal aggregates of beryllium in the sinusoids cellular necrosis has started   By the end of 24 hours these focal aggregates are beginning to fade as the beryllium begins to spread in an ever-widening circle into the

damaged tissue round about   The liver cells begin to disintegrate, and the nuclei either break up into pyknotic fragments or, more commonly, just fade away

The connective tissue of the portal tracts becomes oedematous and infiltrated with round cells   The sinusoids are invaded by large numbers of polymorphonuclear leucocytes   The Kupffer cells show signs of phagocytic activity but no sign of active proliferation

By 48 hours the focal lesions involving the midzone of the lobules are fully developed   In some places eosinophilic masses infiltrated by neutrophils are still present , in others the necrotic material has entered the central veins, leaving behind blood-filled spaces   The reticulum persists until softening is complete and the blood spaces have appeared

In animals that live for 72 hours or more the extent of the liver destruction is very considerable, and a constant feature of the necrosis produced by beryllium is the persistence of a collar of liver cells, as a single layer, round the central veins and portal tracts

Kidney

Within 15 minutes of injection beryllium can be seen in the arcuate vessels and *vasae recti*   It disappears from the former within 15 minutes, but can be seen in the inter-lobular veins up to 30 minutes   Only in isolated cases has it been seen within single glomeruli, and it has not been found in the capillary network surrounding the tubules   Beryllium is, however, found in the medulla of all kidneys examined 30 minutes after injection, lying within the *vasae recti* These vessels, which are arranged in bundles, contain hyaline eosinophilic masses of plasma protein that stain strongly for beryllium   This staining may be seen up to 4 hours after injection and then disappears gradually, so that only in exceptional cases can any beryllium be seen in the *vasae recti* after 24 hours   Unlike the liver, histological changes cannot be detected in the kidney during the first 24 hours, and by the time they do appear all but traces of the injected beryllium have left the kidney   During the second 24-hour period changes take place in some of the proximal convoluted tubules   The tubular epithelium loses its affinity for stains and becomes ragged in outline, while the lumen contains cytoplasmic and nuclear debris, but complete protein casts are not formed at this stage   These lesions are best seen in tissues fixed in Helly's fluid and stained by Heidenhain's azocarmine-aniline blue method   The damaged proximal convoluted tubules with their pale, flattened epithelium and wide lumen superficially resemble distal convoluted tubules, but the damaged tubules can be recognized by their ragged cytoplasm and their position around glomeruli   At 48 hours the changes in the proximal convoluted tubules are well developed   The epithelium of the damaged tubules is no longer columnar, but has become so flattened that the surviving nuclei bulge into the lumen   The protein casts in the lumen of damaged tubules stain brilliantly with azocarmine, and, surrounded by a thin white collar of damaged epithelium, contrast strongly with the purple of undamaged tubules The damaged tubules are found scattered among normal tubules, and a focal lesion which may give the impression of damage to many different tubules is, in fact made up of lesions to the same tubule

At 72 hours the damaged tubules are easier to see owing to the formation of more casts but the number is not increased

The extent and exact site of the damage in individual nephrons has been

investigated by examining serial sections and reconstructing nephrons in plasticine No changes have been detected in the glomeruli The tubular damage starts at the point of origin from the glomerular capsule Damage to the neck of the tubule is always slight, while the most severe damage is in the convoluted part of the proximal convoluted tubule As the straight part is reached the severity of the lesion decreases, and by the time the loop of Henle is reached the epithelium once again appears normal The rest of the nephron is free from pathological change The severe lesions affect only a small number of nephrons, but if apparently normal nephrons are carefully followed in serial section, lesions will commonly be found in the neck and in the convoluted part of the proximal convoluted tubules

Neither the chemical nor histochemical method is able to detect any appreciable quantities of beryllium after the first few hours of an intravenous injection It is possible that the attempted excretion of small quantities of beryllium throughout the whole period of survival may account for the damage, but the localized nature of the lesions suggests that the main injury occurs over a relatively short period of time

Spleen

Diffuse beryllium staining of the red pulp of the spleen appears within 5 minutes of the injection and in 15 minutes is well developed This diffuse staining reaches a maximum within 30 minutes, and then declines slowly, but is still present after 48 hours Concentration of beryllium takes place in the marginal zone between the red and white pulp This is clearly marked after 15 minutes and persists The Malpighian bodies are entirely unstained and clearly outlined by the deeply stained marginal zone This concentration of beryllium is due to the accumulation of the beryllium protein masses within the sinusoids of the marginal zone The same sinusoids are clearly outlined by an intravenous injection of Indian ink into the living animal As the diffuse staining of the red pulp fades, foci of more intense staining develop within the red pulp Histological changes are first seen in the marginal zone between the red and white pulp Small focal lesions develop round the sinusoids Groups of cells become ill-defined and autolysed Within 24 hours these small lesions have spread to involve the whole of the marginal zone, which is now shrunken and empty of large lymphocytes Similar small lesions develop round the sinusoids in the red pulp with destruction of the lymphocytes and other nucleated cells Infiltration of the damaged spleen by granulocytes begins within a few hours of the injection of beryllium

At 72 hours the appearance of the spleen is characteristic of acute beryllium poisoning The cellular white pulp stands out in strong contrast with the structureless red pulp, which is packed with red cells and free of lymphocytes

The surviving nucleated cells in the red pulp are endothelial and reticulum cells, some nests of primitive red and white blood cells, and a relatively large number of mature granulocytes that have infiltrated the damaged tissue The underlying reticular structure remains unaltered

Changes in the white pulp are less extensive It becomes relatively reduced in volume, and the lymphocytes are loosely arranged instead of being packed tightly together

The histological evidence is that beryllium tends to collect in the sinusoids of the spleen, especially in the marginal zones, and round these aggregates of beryl-

hum cellular destruction starts and then spreads rapidly throughout the red pulp
Beryllium never enters the white pulp, and changes here are limited to a reduction
in the number of lymphocytes

Other organs

No changes attributable to beryllium poisoning have been found in the brain,
heart, voluntary muscle, lung, intestine, pituitary, thyroid, parathyroid, pancreas,
ovary, testis, thymus and lymph nodes   Histochemical staining failed to show
any evidence of the accumulation of beryllium in any of these organs, although,
of course, it can be seen within the larger vessels of all organs up to 15 minutes
after an injection   Thirty minutes after the injection of 8 M L D no beryllium
is visible in the lumen of blood vessels except in the kidney

Changes have been seen in the adrenals with shrinkage of the zona glomerulosa
and other less definite changes   These changes are probably associated with the
physiological response to damaged tissues elsewhere

Peripheral blood

The injection of beryllium salts in lethal or sublethal doses produces a sharp
rise in the circulating granulocytes and a fall in circulating lymphocytes   After
sublethal doses the granulocytes return to normal from the fourth day   The
figures from rats injected with lethal doses of beryllium are presented in Table V

TABLE V —*The Effect of a Lethal Dose of Beryllium on the Numbers of Circulating
Granulocytes and Lymphocytes*

	(1000 per c mm)		
	Granulocytes	Lymphocytes	Number of animals
Controls	3 49 $\pm$ 1 64	15 31 $\pm$ 5 47	77
Hours after beryllium			
1 to 8	10 28 $\pm$ 0 67	11 47 $\pm$ 4 86	16
24	14 65 $\pm$ 5 40	10 07 $\pm$ 4 36	21
48	22 87 $\pm$ 4 71	7 82 $\pm$ 3 30	13
72	7 20 $\pm$ 7 25	4 45 $\pm$ 3 26	20

The red blood cells are not affected, nor is there any evidence from a study of
the bone marrow of a disturbance in their maturation processes   The reticulocyte
response to an acute anaemia produced by bleeding was the same in rats acutely
poisoned by beryllium as in normal rats   The assumption is, therefore, that the
changes in the circulating white cells are the result of damage to the other organs,
and this is supported by the evidence of extensive infiltration of the liver and
spleen with granulocytes which starts within a few hours of the injection of
beryllium

The Effect of Subcutaneous and Intraperitoneal Injections of Soluble Beryllium Salts

The ordinary salts of beryllium—sulphate, chloride, lactate, oxyfluoride—are
much less toxic when given by subcutaneous injection   At a dose level of 5
mg /kg beryllium sulphate and lactate will not kill any mice   The same dose of

beryllium as oxyfluoride is fatal, but the pathological picture is not that of beryllium poisoning Death is due to the toxic effects of the fluoride

Both subcutaneous and intraperitoneal injections of beryllium salts give rise to a vigorous local reaction in the tissues, and a chronic inflammatory lesion will develop after a single subcutaneous injection Histochemical staining shows that the beryllium becomes fixed to the tissues at the site of the injection, and in the rabbit many remain for as long as 3 months after an intradermal injection The reaction takes place round this fixed beryllium The salts of Ca, Mg, and Al do not produce any such local reactions

If the beryllium is injected subcutaneously as a solution of sodium beryllium tartrate its toxicity is much greater than an equivalent dose of beryllium as lactate or sulphate Thus at a dose of 5 mg /kg no mice died after the subcutaneous injection of beryllium lactate or sulphate, while all mice died after injection of sodium beryllium tartrate The pathological picture in these animals was characteristic of beryllium poisoning

The beryllium in the sodium beryllium tartrate is not in an ionized form, and a much greater proportion gets carried away into the circulation This was shown in an experiment with a series of mice injected with 200 µg beryllium Some received beryllium lactate and others sodium beryllium tartrate Mice were killed at intervals after the injection and the liver, spleen and kidneys removed and examined spectrographically Within 15 minutes there was the equivalent of 1000 parts beryllium per million parts of dry weight of liver, spleen and kidney in the animals receiving sodium beryllium tartrate, while no beryllium at all could be detected within 2 hours in the animals receiving beryllium lactate, and only traces were present 18 hours later

Apparently ionic beryllium has the power of attaching itself rapidly and firmly to tissue protein It also attaches itself to serum protein after intravenous injection The histochemical staining always shows the beryllium to be present in amorphous masses within sinuses, etc , and these masses have all the other staining properties of protein

If beryllium is added to serum *in vitro* it rapidly becomes firmly attached, and an ultrafiltrate of serum to which beryllium has been added in a concentration of 50 µg /ml contains no beryllium at all Ordinary salts of beryllium are precipitated by sodium phosphate, but if the beryllium is first added to serum and then sodium phosphate is added, no precipitate forms Beryllium salts, such as the lactate, do not precipitate serum proteins *in vitro*

Little is known about this peculiar affinity of beryllium for protein

Although this attachment to protein will protect beryllium from precipitation as phosphate, it does not in any way reduce its toxicity If beryllium lactate is first added to serum and left for periods up to 24 hours, this treated serum has all the toxic properties of the added beryllium

DISCUSSION

This peculiar affinity of beryllium for protein suggested the possibility that the syndrome of beryllium poisoning might be attributable to the destruction or inhibition of some specific enzyme as a result of beryllium becoming attached thereto

Early in the course of the work it was discovered that rabbits died from beryllium poisoning with blood sugar levels of 20 mg /100 ml or less The

terminal convulsions were almost certainly hypoglycaemic in nature   Rats also died with low blood sugar levels

It is hoped to report the full biochemical changes elsewhere, but it can be stated here that, as yet, no evidence has been found of any specific disturbance of the carbohydrate metabolism in the early stages of beryllium poisoning   Blood sugar levels, sugar tolerance curves, liver glycogen levels and blood lactic acid levels and tolerance curves were all within normal limits in the first 24 hours of poisoning   Disturbances did occur, but only at a time when they could be accounted for by the amount of the destruction of liver tissue that had taken place   The animals apparently die from an acute liver failure   This belief is supported by the experimental proof that repeated intravenous injections of glucose will prolong the life of fatally poisoned animals

The destruction of the liver is apparently the result of an accumulation of beryllium, in the sinusoids which takes place within the first few hours of the intravenous injection of beryllium   Foci of necrosis start round the deposited beryllium and extend by enlargement and confluence to form a midzonal necrosis Opie (1925), from a study of man and animals, concluded that focal necrosis was the antecedent condition for the development of midzonal necrosis   This same train of events has been seen after poisoning with a wide variety of agents—bacteria, bacterial toxins, vegetable toxins and metallic salts   As a rule the focal necrosis and subsequent zonal necrosis is centrilobular rather than midzonal   At first sight it appears paradoxical that the liver should accumulate within its sinusoids a substance such as beryllium, which then proceeds to destroy the liver tissue and so kill the animal   This appears less peculiar if it is assumed that beryllium, by attaching itself to protein, produces some foreign colloidal material   Foreign material of a colloidal or particulate nature is normally segregated in the sinusoids of the liver and spleen, while hardly any can be found in the kidney, stomach, intestines, central nervous system or voluntary muscles (Drinker and Shaw, 1921 , Lund, Shaw and Drinker, 1921, Drinker , Shaw and Drinker, 1923 , Voigt, 1914a, 1914b, 1915, 1918, Duhamel, 1919 , Gye and Purdy, 1922)

Once these materials have been removed from the general circulation and collected in the liver sinusoids they are either taken up by the Kupffer cells or absorbed by the liver cells   It is presumably the attempt to absorb the beryllium that damages the hepatic cells   The diversity of agents that produce liver disease has led to a search for a final common path through which they exert their toxic effect   Though it has been suggested that some susceptible enzyme system might supply this path, no such system has yet been identified   An alternative hypothesis is that masses of foreign material in the liver sinusoids produce focal necrosis as a result of vascular stasis   Though this view has the authority of Mallory, the condition of stasis has yet to be adequately demonstrated   In the case of beryllium poisoning the first change seems to be an alteration in the cell permeability, resulting in cellular oedema   Foci of cells close to aggregates of beryllium then break down, and necrosis has started   This suggests that beryllium may affect the permeability of the cell membrane

The lesions in the spleen apparently have an origin similar to those of the liver, for the cells that first show evidence of damage are those around the focal concentrations of beryllium in the sinuses of the marginal zone and in the red pulp   Necrosis and lysis spread until all but the reticulum itself is destroyed

The cells of the hepatic parenchyma and those of the red pulp of the spleen bear no functional resemblance to one another   This suggests that the action of beryllium is a general one, and at the present time little more can be said than that beryllium or beryllium protein complexes irreversibly damage some system common to the surfaces of certain types of cell

The renal lesions are not so readily accounted for   After large doses of beryllium intravenously the metal can only be detected in the *vasae recti*, and none can be seen within or around the tubules that subsequently show the damage   Chemical analysis shows that very little beryllium can be found in the whole kidney, and very little beryllium is excreted in the urine   However, before dismissing the possibility of direct damage to the tubules by beryllium, it must be remembered that no beryllium can be detected histochemically even in the liver after a single lethal dose   In other words, liver necrosis can result from the presence of amounts of beryllium too small to be detected histochemically   It is possible that in the first few minutes after injection some tubules come into direct contact with beryllium, but that the kidney reacts in a different way to the presence of this irritant   The liver collects the beryllium, but the kidney, possibly by means of the vascular shunt described by Trueta, Barclay, Daniel, Franklin and Prichard (1947), protects itself from further damage and beryllium has no means of accumulating within it   The occasional case where cortical necrosis develops may be the result of an overaction of this shunt

Work with beryllium *in vitro* may often be complicated by the formation of the very insoluble beryllium phosphate   Although there is theoretically enough phosphate in the circulating blood to precipitate the beryllium injected as a single lethal dose, such precipitation and inactivation does not take place, presumably because of the protective role of the protein   On the other hand, when beryllium is given by mouth the inorganic phosphate in the gut is able to prevent the absorption of any significant quantity of beryllium   As Branion, Kay and Guyatt (1931) showed, feeding beryllium to rats will produce rickets by depriving the animals of phosphate, and these animals die with a gross phosphate deficiency   We have confirmed these observations and produced beryllium rickets, but rats dying during these experiments show none of the tissue changes that are characteristic of beryllium poisoning   Furthermore, the tissues of these animals, when examined spectrographically after 3 months on a diet containing 2 5 per cent beryllium as a soluble salt, contain only traces of the metal

CONCLUSIONS

Beryllium ions have the property of reacting rapidly with certain tissue proteins, including plasma protein   The complex so formed is of an unknown nature   It is of sufficient stability to protect beryllium from precipitation by phosphate ions   Such precipitation is the fate of beryllium ions reaching the alimentary canal, with the result that beryllium leaves the body without harming it except in so far as it may deprive it of phosphates where the amount of beryllium is large and its administration is continued for long periods

The beryllium-protein complex is probably well held together because of the property beryllium possesses of forming co-valent compounds   The protein is not obviously denatured (precipitated) by the union with beryllium   At the same time, the original toxicity of the beryllium ions is fully maintained

In the subcutaneous tissues beryllium attaches itself to protein forming part of the permanent tissue elements, for after a single injection beryllium may remain *in situ* for many weeks   Round the beryllium chronic inflammatory lesions develop and persist presumably as a response to the continual slight tissue destruction produced by the presence of beryllium   Because of this affinity for the fixed structures, beryllium injected into tissues or inhaled tends to remain localized   After a dose is administered in either of these ways the animal may die from acute beryllium poisoning if enough beryllium escapes from the site of injection to overwhelm the liver   In other cases the animal may die from the acute inflammatory effects produced by the beryllium, as, for example, when a large dose is inhaled and a secondary pneumonia develops

When beryllium ions are injected into the circulation, they attach themselves to plasma protein and not to the endothelial lining of the vessel walls   The beryllium-protein complex is then removed from circulation by the liver, and by doing this the liver sows the seeds of its own destruction, for before it can excrete the beryllium, the latter has started to destroy the hepatic cells   Where the dose is large enough destruction may proceed almost to completion, but it can be halted after the injection of sublethal doses

The exact nature of the change produced by beryllium on liver cells is unknown   It interferes with some intimate cellular mechanism, and other cells, such as the lymphocytes of the spleen, are also killed by beryllium   Vascular endothelium and neutrophil leucocytes appear to be more resistant to the action of beryllium

Beryllium ions are poisonous to all living cells when brought into intimate contact with them   Their affinity for tissue protein and their insolubility in the presence of phosphate means that their toxicity to the whole animal is determined to a very large extent by the route of their administration

SUMMARY

1   The lethal dose of beryllium injected intravenously into rabbits, rats and mice as solutions of ionized salts is 0 5 to 1 mg per kg (20 micromol)

2   Death takes place 1 to 3 days later and is the result of acute liver necrosis

3   Neither the toxic properties of the anionic component nor the acidity of the solutions of the beryllium salts are responsible for these deaths

4   By histochemical methods it is possible to see beryllium collecting in the liver sinusoids and initiating the necrotic changes

5   Beryllium ions rapidly attach themselves to protein, and in this form are carried to the liver after intravenous injection   After subcutaneous injection, on the other hand, they tend to remain for long periods attached to tissue proteins

6   Beryllium salts produce inflammatory changes in tissues after injection or inhalation, but as most of the beryllium is fixed to the tissues, death from liver necrosis only follows the injection of very large doses

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SOME EFFECTS OF ANTERIOR PITUITARY EXTRACT ON LIVER METABOLISM [*]

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BURN AND LING (1930) were the first workers to note the effect of injections of extracts of the anterior lobe of the pituitary in producing a ketonuria in the rat   Since that time much work has been done on various aspects of this finding, but little on the effects of such extracts on liver metabolism   On evidence of an indirect nature, Collip, Kutz, Long, Thomson, Toby and Selye (1935) and Mirsky (1936) have concluded that the ketogenic fraction of such extracts produces its effects by acting solely on the liver   Shipley (1944) found that the addition of a crude extract of the anterior lobe to rat liver slices incubated in serum produced a 46 per cent and a 27 per cent increase in ketone body production in the case of the fed and fasted animal respectively   No experiments appear to have been done prior to 1941 on the livers of animals treated with such extracts   Livers of animals so treated are characterized by an intense deposition of fat and it thus seemed worthwhile to examine their metabolic behaviour

[*] This communication forms part of a thesis submitted to the University of Melbourne in 1943

Animals

Well-fed male guinea-pigs of about 500 g body-weight were used throughout

Anterior Pituitary Extracts

Fresh beef anterior lobes were finely minced and ground with saline at room temperature for one hour The extracts were then centrifuged and stored at $+1°$ until required No extract was kept longer than two days The volume of the extract was so adjusted that 1 ml was equivalent to 0 5 g of fresh gland

Animal treatment

All animals injected with anterior pituitary extract were given either 1 0 or 3 0 ml subcutaneously over the dorsal region, and were sacrificed at varying time intervals after injection

Manometric and analytical techniques

The methods followed were as described in an earlier communication (Ennor, 1942)

RESULTS

The behaviour of livers from untreated guinea-pigs is illustrated by the results in Table I

These results have, for the most part, been published in the earlier communication (Ennor, 1942), and are reproduced here for the purpose of comparison with those obtained in fatty livers, which are given in Table II

TABLE I —*Oxygen Consumption and Acetoacetate Production in Livers of Normal, Well-fed Guinea-pigs*

Liver fat (g %)	Control—no substrate				Sodium butyrate—0 01 M			
	Q_O	Q_{ac}	$Q_{O_2}^c$	Q_{ac}^c	Q_O	Q_{ac}	$Q_{O_2}^c$	Q_{ac}^c
5 4	6 1	0 2	7 6	0 2	6 6	1 6	8 3	2 0
6 5	5 0	0 2	6 7	0 2	7 4	1 2	9 9	1 6
5 3	5 1	0 1	6 4	0 1	7 9	1 4	9 9	1 8
6 S	3 7	0 1	5 0	0 1	6 2	1 5	8 2	2 0
6 5	4 9	0 1	6 4	0 1	8 8	2 7	11 6	3 6
6 4	3 9	0 0	4 7	0 0	7 8	1 2	9 5	1 5
6 5	4 3	0 2	5 6	0 3	8 1	1 8	10 5	2 4
5 3	6 6	—	8 3	—	11 6	—	14 5	—
S 2	4 4	0 0	5 9	0 0				
6 2	6 3	0 2	S 0	0 3				
6 6	4 4	0 0	5 7	0 0				
6 S	4 6	0 2	6 2	0 3				
6 9	6 2	0 0	8 4	0 0				

The quotients Q_{O_2} and Q_{ac} are defined respectively, as the number of c mm of O_2 and number of c mm CO_2 (arising from acetoacetate)/mg of dry weight of liver slice/hr

The quotients $Q_{O_2}^c$ and Q_{ac}^c are defined, respectively, as the number of c mm of O_2 and number of c.mm CO_2 (arising from acetoacetate)/mg of *fat free* dry weight of liver slice/hr

TABLE II—*Oxygen Consumption and Acetoacetate Production in Livers from Guinea-pigs Treated with Anterior Pituitary Extract (A P E)*

Time after injection (hr)	Dose APE g fresh gland	Liver fat (g %)	Control—no substrate				Sodium butyrate—0 01 M			
			Q_{O_2}	Q_{ac}	$Q^c_{O_2}$	Q^c_{ac}	Q_O	Q_{ac}	$Q^c_{O_2}$	Q^c_{ac}
3	1 5	6 9	4 4	0	5 7	0	10 7	2 3	13 7	3 0
3	1 5	4 7	5 2	0 3	7 3	0 4	9 6	1 9	11 6	2 3
3	1 5	9 4	3 4	0 4	4 8	0 5	7 0	2 2	10 4	3 2
4	1 5	7 5	3 9	0 1	5 7	0 2	9 5	2 5	12 5	3 3
5	1 5	10 4	5 8	0 7	8 6	1 0	9 5	3 1	14 3	4 6
5	0 5	8 1	4 5	0 2	6 2	0 3				
5	0 5	6 9	3 7	0 1	4 9	0 2				
10	0 5	7 4	5 4	0 4	7 2	0 5	11 6	3 1	15 4	4 1
11	0 5	15 5	4 6	0 4	9 2	0 7	9 7	2 5	19 4	5 0
11	1 5	10 3	4 3	0 2	6 3	0 3	8 2	2 1	12 1	3 1
11	1 5	12 4	3 8	0 1	6 7	0 1	6 6	1 8	10 4	2 8
11	1 5	7 0	4 6	0 4	6 1	0 5	10 4	2 9	14 0	3 8
17	0 5	9 1	5 7	0 2	7 8	0 3				
18	1 5	19 7	3 4	—	7 4	—	6 9	3 1	15 0	6 7
19	1 5	13 3	5 4	0 5	9 0	0 9	8 2	2 3	13 6	3 8
24	1 5	19 9	4 2	0 3	9 5	0 6	6 4	1 6	12 8	3 1
24	1 5	20 0	4 5	0 3	8 8	0 6	7 1	2 1	13 7	4 0
24	1 5	15 0	4 9	0 4	8 4	0 7	7 0	2 0	13 5	3 5
32	1 5	13 2	5 4	0 8	9 0	1 4	8 6	2 2	14 3	3 7

In the absence of added substrate (Table II) there is no obvious departure from normality as far as the Q_{O_2} values are concerned The Q_{ac} values, however, show a definite increase over the comparable figures from Table I When the results calculated on the basis of fat-free dry weight are considered, there is evidence of an increase in the $Q^c_{O_2}$, whilst the Q^c_{ac} values are, of course, increased to a still greater degree

Unfortunately the experiments carried out at each time interval are not sufficiently numerous to establish the sequence of events within the liver in relation to time Broadly, however, it may be said that the effects of anterior pituitary extract are detectable three hours after injection Although the fat content is not unequivocally changed, the Q^c_{ac} values are definitely in excess of normal This result would seem to suggest that the first effect of the anterior pituitary extract is to increase the rate of ketogenesis, and thus to lend support to Shipley's (1944) conclusion (cf Best and Campbell (1938)) As the time interval between injection and sacrifice is increased, the effect of anterior pituitary extract on liver fat and $Q^c_{O_2}$ and Q^c_{ac} becomes more pronounced Consistently maximal figures for these are to be seen at the 24-hour interval, which was the longest period investigated

There is also a difference in the behaviour of the livers from Table I and Table II in their reactions towards added substrate These differences become apparent in Table III, where the difference between the quotients (expressed on a fat-free dry weight basis) for the oxygen consumption and acetoacetate production in the absence and presence of added substrate is shown

<div align="center">EXPERIMENTAL</div>

Animals

Well-fed male guinea-pigs of about 500 g body-weight were used throughout

Anterior Pituitary Extracts

Fresh beef anterior lobes were finely minced and ground with saline at room temperature for one hour   The extracts were then centrifuged and stored at $+1^\circ$ until required   No extract was kept longer than two days   The volume of the extract was so adjusted that 1 ml was equivalent to 0 5 g of fresh gland

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The behaviour of livers from untreated guinea-pigs is illustrated by the results in Table I

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	Q_{O_2}	Q_{ac}	$Q_{O_2}^c$	Q_{ac}^c	Q_{O_2}	Q_{ac}	$Q_{O_2}^c$	Q_{ac}^c
5 4	6 1	0 2	7 6	0 2	6 6	1 6	8 3	2 0
6 5	5 0	0 2	6 7	0 2	7 4	1 2	9 9	1 6
5 3	5 1	0 1	6 4	0 1	7 9	1 4	9 9	1 8
6 8	3 7	0 1	5 0	0 1	6 2	1 5	8 2	2 0
6 5	4 9	0 1	6 4	0 1	8 8	2 7	11 6	3 6
6 4	3 9	0 0	4 7	0 0	7 8	1 2	9 5	1 5
6 5	4 3	0 2	5 6	0 3	8 1	1 8	10 5	2 4
5 3	6 6	—	8 3	—	11 6	—	14 5	—
S 2	4 4	0 0	5 9	0 0				
6 2	6 3	0 2	S 0	0 3				
6 6	4 4	0 0	5 7	0 0				
6 S	4 6	0 2	6 2	0 3				
6 9	6 2	0 0	8 4	0 0				

The quotients Q_{O_2} and Q_{ac} are defined, respectively, as the number of c mm of O_2 and number of c.mm CO_2 (arising from acetoacetate)/mg of dry weight of liver slice/hr

The quotients $Q_{O_2}^c$ and Q_{ac}^c are defined, respectively, as the number of c mm of O_2 and number of c.mm CO_2 (arising from acetoacetate)/mg of *fat free* dry weight of liver slice/hr

TABLE II —*Oxygen Consumption and Acetoacetate Production in Livers from Guinea-pigs Treated with Anterior Pituitary Extract (A P E)*

Time after injection (hr)	Dose A P E g fresh gland	Liver fat (g %)	Control—no substrate				Sodium butyrate—0 01 M			
			Q_{O_2}	Q_{ac}	$Q^c_{O_2}$	Q^c_{ac}	Q_{O_2}	Q_{ac}	Q^c_O	Q^c_{ac}
3	1 5	6 9	4 4	0	5 7	0	10 7	2 3	13 7	3 0
3	1 5	4 7	5 2	0 3	7 3	0 4	9 6	1 9	11 6	2 3
3	1 5	9 4	3 4	0 4	4 8	0 5	7 0	2 2	10 4	3 2
4	1 5	7 5	3 9	0 1	5 7	0 2	9 5	2 5	12 5	3 3
5	1 5	10 4	5 8	0 7	8 6	1 0	9 5	3 1	14 3	4 6
5	0 5	8 1	4 5	0 2	6 2	0 3				
5	0 5	6 9	3 7	0 1	4 9	0 2				
10	0 5	7 4	5 4	0 4	7 2	0 5	11 6	3 1	15 4	4 1
11	0 5	15 5	4 6	0 4	9 2	0 7	9 7	2 5	19 4	5 0
11	1 5	10 3	4 3	0 2	6 3	0 3	8 2	2 1	12 1	3 1
11	1 5	12 4	3 8	0 1	6 7	0 1	6 6	1 8	10 4	2 8
11	1 5	7 0	4 6	0 4	6 1	0 5	10 4	2 9	14 0	3 8
17	0 5	9 1	5 7	0 2	7 8	0 3				
18	1 5	19 7	3 4	—	7 4	—	6 9	3 1	15 0	6 7
19	1 5	13 3	5 4	0 5	9 0	0 9	8 2	2 3	13 6	3 8
24	1 5	19 9	4 2	0 3	9 5	0 6	6 4	1 6	12 8	3 1
24	1 5	20 0	4 5	0 3	8 8	0 6	7 1	2 1	13 7	4 0
24	1 5	15 0	4 9	0 4	8 4	0 7	7 0	2 0	13 5	3 5
32	1 5	13 2	5 4	0 8	9 0	1 4	8 6	2 2	14 3	3 7

In the absence of added substrate (Table II) there is no obvious departure from normality as far as the Q_{O_2} values are concerned   The Q_{ac} values, however, show a definite increase over the comparable figures from Table I   When the results calculated on the basis of fat-free dry weight are considered, there is evidence of an increase in the $Q^c_{O_2}$, whilst the Q^c_{ac} values are, of course, increased to a still greater degree

Unfortunately the experiments carried out at each time interval are not sufficiently numerous to establish the sequence of events within the liver in relation to time   Broadly, however, it may be said that the effects of anterior pituitary extract are detectable three hours after injection   Although the fat content is not unequivocally changed, the Q^c_{ac} values are definitely in excess of normal   This result would seem to suggest that the first effect of the anterior pituitary extract is to increase the rate of ketogenesis, and thus to lend support to Shipley's (1944) conclusion (cf Best and Campbell (1938))   As the time interval between injection and sacrifice is increased, the effect of anterior pituitary extract on liver fat and $Q^c_{O_2}$ and Q^c_{ac} becomes more pronounced   Consistently maximal figures for these are to be seen at the 24-hour interval, which was the longest period investigated

There is also a difference in the behaviour of the livers from Table I and Table II in their reactions towards added substrate   These differences become apparent in Table III, where the difference between the quotients (expressed on a fat-free dry weight basis) for the oxygen consumption and acetoacetate production in the absence and presence of added substrate is shown

TABLE III —*Increments in Oxygen Consumption and Acetoacetate Production in Livers from Normal and Anterior Pituitary Extract-treated Guinea-pigs arising as a Result of Added Substrate*

Normal		Treated with anterior pituitary extract	
$\Delta Q^c_{O_2}$ *	ΔQ^c_{ac} †	$\Delta Q^c_{O_2}$ *	ΔQ^c_{ac} *
0 7	1 8	8 0	3 0
3 2	1 4	4 3	1 9
3 5	1 7	5 6	2 7
3 2	1 9	6 8	3 1
5 2	3 5	5 7	3 6
4 8	1 5	8 2	3 6
4 9	2 1	10 2	4 3
6 2	—	5 8	2 8
		3 7	2 7
		7 9	3 3
		7 6	—
		4 6	2 9
		3 3	2 5
		4 9	3 4
		5 1	2 8
		5 3	2 3

* $\Delta Q^c_{O_2}$ = $Q^c_{O_2}$ in presence of 0 01 M sodium butyrate—$Q^c_{O_2}$ in absence of substrate

† ΔQ^c_{ac} = Q^c_{ac} in presence of 0 01 M sodium butyrate—Q^c_{ac} in absence of substrate

There is evidence that, in the presence of 0 01 M sodium butyrate, the livers from the pituitary-treated animals show a greater oxygen uptake and a greater acetoacetate production than do the livers from the untreated animals

In view of the general similarity of behaviour of the fatty liver arising from treatment with anterior pituitary extract to that following poisoning with carbon tetrachloride, it is of interest to compare the mean quotient values for oxygen consumption and acetoacetate production obtained from normal anterior pituitary extract-treated, and carbon tatrachloride-treated animals   Table IV illustrates the results obtained

TABLE IV —*Mean Values for Quotients Obtained from Livers of Normal Anterior Pituitary Extract and CCl_4-treated Guinea-pigs in the Absence of Added Substrate*

	Q_{O_2}	Q_{ac}	$Q^c_{O_2}$	Q^c_{ac}
Normal	5 0	0 1	6 5	0 1
Anterior pituitary extract (> 10% fat)	4 6	0 4	8 3	0 7
CCl_4 (> 10% fat)	6 1	0 3	11 5	0 6

In this table the mean figure for the normal values has been calculated from the figures given in Table I, and that for anterior pituitary extract-treated animals from Table II   In the latter case, only those values obtained with livers with more than 10 per cent of fat have been selected for calculation of the means   The results for the carbon tetrachloride livers have been taken from

Tables II and III in a previous publication of the author (Ennor, 1942), and here again only the figures from those animals whose livers contained more than 10 per cent of fat have been included in the calculation of the means This selection of results has been made to ensure that only those livers with fat content definitely in excess of the normal upper limit are included for comparison

As far as the Q_O means are concerned, the values for the normal and the anterior pituitary extract-treated livers are substantially the same, and since these figures are based on the dry weight of the liver slices, it is clear that, as the fat content of the anterior pituitary extract livers is in excess of 10 per cent, the actual respiring tissue must be utilizing more oxygen than is the case in the normal liver This is substantiated by the Q_O^c figures, which show that the anterior pituitary extract-treated livers have a definitely higher oxygen consumption In the case of those livers from animals treated with carbon tetrachloride, the oxygen consumption is increased irrespective of the method of calculation Similar remarks may be applied to the acetoacetate production from both types of fatty livers It is therefore concluded that an influx of fat into the liver, due either to treatment with carbon tetrachloride or anterior pituitary extract is followed by an increase in the oxygen consumption and the acetoacetate production There does, however appear to be a difference between the behaviours of these two types of fatty liver It will be noted from Table IV that these livers have very nearly the same capacity for production of ketones, but that there seems to be a real difference in their ability to consume oxygen Thus, the livers from the animals treated with anterior pituitary extract consume only two-thirds of the oxygen that is consumed by the livers from animals treated with carbon tetrachloride

DISCUSSION

The results of this investigation are of interest, particularly in view of the recent work of Campbell and Davidson (1949) These workers have also observed the increase in oxygen consumption and acetoacetate production of the livers from animals injected with anterior pituitary extract They concluded that there was evidence of a pituitary factor which caused enhanced activity of the fat metabolizing centres Such a conclusion is in agreement with the results reported here, since there is evidence that the pituitary fatty liver responds to added substrate by consuming more oxygen and producing more acetoacetate than does the normal liver

Oxygen is necessary for the oxidation of fat, and if this is being oxidized rapidly and in amounts greater than normal, as is indicated by the high Q_{ac}^c values in all animals having fatty livers, then oxygen is necessary in amounts greater than normal in proportion to the amounts of ketone bodies being produced This generalization agrees with the conditions existing in the livers of animals treated with anterior pituitary extract and with carbon tetrachloride It is only when the results obtained with these types of fatty livers are compared that a difference in the degree of response is obvious In the case of those animals treated with anterior pituitary extract, the increase in oxygen uptake is not of the same magnitude as in the case of the carbon tetrachloride fatty liver, though the acetoacetate production is comparable Since the oxygen uptake is equal to the sum of the oxygen necessary for the oxidation of fat, plus that necessary for the oxidation of other substrates, it would seem that, in the case of the pitui-

tary fatty liver, the extra amount of oxygen necessary for the oxidation of fat is obtained at the expense of that necessary for the oxidation of other substrates The net result is that oxygen is made available for the oxidation of fat without a marked increase in the total uptake being apparent Such an hypothesis suggests that the anterior pituitary extract, in addition to producing an increase in liver fat, may also inhibit other enzyme mechanisms dependent upon oxygen There is no indication from the present work as to what, if any, mechanisms are inhibited, but that inhibition is possible is suggested by the work of Colowick, Cori and Slein (1947) On the other hand, it is possible that the difference may be due to an overall increase in metabolism from carbon tetrachloride-treated animals It is hoped that work at present in progress in this laboratory may give further information on this problem

SUMMARY

1 The effects of injections of crude extracts of the anterior pituitary gland on the liver metabolism of the guinea-pig have been investigated

2 Associated with the increase in liver fat of animals so treated, there is an increase in the oxygen consumption and acetoacetate production

3 Comparison of the results with those obtained in the fatty liver following treatment with carbon tetrachloride indicates a difference in response which may be due to an inhibitory influence of the extracts on oxidative processes other than those concerned with fat metabolism

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SEROLOGICAL PROTECTION AGAINST THE "FATTY LIVER"-PRODUCING EFFECT OF ANTERIOR PITUITARY EXTRACTS *

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IT was noted by Best and Campbell (1938) that the injection of extracts of the anterior lobe of the pituitary gland into guinea-pigs was followed by a marked rise in liver fat in the first 24 hours   It was also noted that a further rise, although less marked, occurred in the next 24-hour period, but on the third day the liver fat actually decreased in spite of continued injection of the extract   No explanation of this finding was offered by these workers, and it occurred to the present authors that these experiments bore a close analogy to those which were described in a communication dealing with the action of antidiabetogenic sera (Ennor and Singer, 1941)   It was thought that the probable explanation of Best and Campbell's findings was that the animals had developed antibodies to the fat-producing factor, and that, after the initial accumulation of fat had been oxidized or otherwise removed, the antibody reaction was sufficiently great to negate the activity of the extract

This communication gives the results of investigations into this hypothesis

EXPERIMENTAL

Anterior pituitary extracts

All extracts used were prepared as described by Ennor (1949)

Preparation of anti-serum

Adult English hutch rabbits were used throughout   Each animal was given an intravenous injection of 1 ml of the anterior pituitary extract daily for the first three days, and thereafter 1 ml on every third day until such time as the antibody titre was considered sufficiently high   The animals were then bled with aseptic precautions, and after clotting the serum was separated and stored at $+1°$ for use   The titre of the serum was tested by complement fixation with anterior pituitary extract diluted 1/10, and at the time of use was 1/320   Prior to injection, antibodies specific to ox serum proteins were removed by precipitation with ox serum

Manometric and analytical techniques

The methods followed were as described in an earlier communication (Ennor, 1942)

27

Animals

Well-fed male guinea-pigs of about 500 g body-weight were used throughout

RESULTS

Control series

Several control experiments were carried out to determine the effect of normal rabbit serum and immune serum   In the case of those animals receiving serum, 6 0 ml were administered intraperitoneally 18 hours before sacrifice   Such injections apparently exert no influence either on the fat content or on the reaction towards added substrate (Table I)

TABLE I —*Oxygen Consumption and Acetoacetate Production of Livers from Guinea-pigs Untreated and Treated with Normal Serum and Immune Serum*

Liver fat (g %)	Control—no substrate		Na butyrate—0 01 M	
	$Q_{O_2}^{c}$ [*][*]	Q_{ac}^{c} [*]	$Q_{O_2}^{c}$ [*]	Q_{ac}^{c} [*]
Normal controls				
5 4	5 8	0 2	13 2	3 1
6 4	4 7	0	9 5	1 5
5 7	7 4	0 2	12 8	3 6
Normal serum controls				
5 4	6 8	0 1	11 1	2 7
5 8	8 2	0	—	—
4 5	8 0	0	12 1	3 2
Immune serum controls				
6 0	8 0	0 5	14 4	3 7
3 4	6 6	0 2	13 1	3 1

* The quotients $Q_{O_2}^{c}$ and Q_{ac}^{c} are defined, respectively, as the number of c mm of O_2 and number of c mm CO_2 (arising from acetoacetate) /mg of fat free dry weight of liver slice/hr

Anterior pituitary extract treated series

Here the animals were given a single subcutaneous injection of anterior pituitary extract which was equivalent to 1 5 g of fresh gland   In the case of animals receiving serum in addition to the anterior pituitary extract, this was given 8 hours prior to the extract   The animals were then sacrificed as before, making a total of 18 hours' survival after injection of serum

The results are shown in Table II, where " absorbed serum " refers to immune serum from which antibodies were removed by absorption with anterior pituitary extract before injection   As is well known, the injection of anterior pituitary extract is followed by an increase in liver fat, and this is uninfluenced by the injection of normal serum prior to the anterior pituitary extract   The metabolic behaviour of the livers from the two groups is similar   If, however, immune serum is substituted for normal serum, the injection of anterior pituitary extract does not result in any change of the liver fat content , complete protection is thus afforded   The oxygen consumption appears to be rather larger than normal, and the acetoacetate production definitely so   An explanation of this effect

TABLE II.—*Oxygen Consumption and Acetoacetate Production of Livers from Guinea-pigs Treated with Anterior Pituitary Extract and Anterior Pituitary Extract + Serum*

Liver fat (g %)	Control—no substrate		Na butyrate—0 01 M	
	$Q_{O_2}^c$ *	$Q_{ac.}^c$ *	$Q_{O_2}^c$ *	Q_{ac}^c *
Anterior pituitary extract only				
15 4	9 2	0 7	19 4	5 0
10 3	6 3	0 3	12 1	3 1
Anterior pituitary extract + normal serum				
11 9	8 6	0 1	11 1	2 7
10 6	7 6	0 4	12 1	2 3
Anterior pituitary extract + immune serum				
6 3	6 4	0 2		
6 3	7 0	0 3		
6 0	6 9	0 4	12 4	3 0
5 7	6 5	0 3	13 0	3 4
6 5	6 8	0 2	12 7	2 8
7 0	8 4	0 6	13 7	3 8
Anterior pituitary extract + absorbed serum				
13 2	11 1	—	11 3	3 1

* The quotients $Q_c^{O_2}$ and Q_{ac}^c are defined, respectively, as the number of c.mm of O_2 and number of c.mm CO_2 (arising from acetoacetate) /mg of fat free dry weight of liver slice/hr

will have to await further work on the problem The single experiment at the foot of Table II indicates that the protection gained by the prior injection of immune serum is completely lost if the serum is absorbed with crude extract before injection

SUMMARY

It is possible to inhibit the transference of fat from body depots to the liver, which follows subcutaneous injection of anterior pituitary extract in guinea-pigs, by prior administration of immune serum

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THE ISOLATION OF ANTIBIOTICS PRODUCED BY *PROACTINOMYCES (NOCARDIA) GARDNERI*

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GARDNER AND CHAIN (1942) described some of the properties of a basic antibiotic produced by a chance contaminant on an agar plate   The organism was identified as a *Proactinomyces* by Waksman, and it was given the name *Proactinomyces (Nocardia) gardneri*   The antibiotic was called proactinomycin

McKegney and Kent (unpublished, 1945) found that in surface culture the best yield of the antibiotic was obtained when the organism was grown on a potato dextrose medium containing 0 25 per cent agar   The extraction process of Gardner and Chain, which utilized the fact that the free base was more soluble than its salts in organic solvents, was modified by Abraham and Kent (unpublished, 1945) for large scale work

The crude product was purified by partition between dilute hydrochloric acid and butyl alcohol-benzene mixtures on a silica gel column (Philpot, unpublished, 1946)   No inhomogeneity was detected in the product obtained from the column

Gardner and Chain found that the active substance could be precipitated from aqueous solution by base precipitants such as picric acid, picrolonic acid, flavianic acid, Reinicke acid and helianthic acid   The free base was slightly soluble in water and soluble in alcohol, acetone, ether, chloroform and benzene, and its salts with mineral acids were soluble in water, alcohol and acetone   Proactinomycin was stable in aqueous solution between pH 2 and 8, but was inactivated at room temperature at a pH greater than 10, or by boiling at pH 2   Philpot (unpublished, 1946) detected one titratable group with an approximate pK of 9 On mild treatment of proactinomycin with alkali she obtained (1) dimethylamine, (2) a base with an empirical formula $C_{18}H_{29}NO_5$, and (3) a neutral fraction with empirical formula $C_6H_9O_2$ from which an osazone could be obtained

Heatley (unpublished, 1948) demonstrated in two counter-current distribution experiments that the product used in previous experiments was composed of at least three antibacterial components   This discovery prompted a reinvestigation of the properties of proactinomycin

EXPERIMENTAL

Production of material

An attempt was made to select a high-yielding strain of the organism, and 11 single-spore isolates were tested by the cross streak method against *Staph aureus*   No significant difference in the dimensions of the inhibitory zones was noted

Stationary culture —Part of the proactinomycin for these experiments was

prepared by the method of McKegney and Kent (unpublished, 1945) in rectan gular ceramic vessels holding about 1 litre (Abraham, Chain, Fletcher, Florey Gardner, Heatley and Jennings, 1941) which were incubated at 24° C for 9 day at which time maximum activity was present, as measured by the cylinder-plat assay with *Staph aureus* as the test organism (Heatley, 1944) The pH of th medium remained between 6 8 and 7 2, and the slight inconsistent changes in could not be correlated with the level of activity The medium was harveste after 9 days and squeezed through muslin or lint to remove the mycelium an break up the agar into small pieces

Deep culture —A preliminary experiment showed that maximum antibacteria activity was produced in aerated culture after a shorter period of incubation tha was necessary for stationary cultures, although the final level obtained by eithe method was approximately the same The medium was the same as that use for stationary culture except that no agar was included

Some proactinomycin was prepared in 10-litre bottles set up as described b Stansly, Schlosser, Ananenko and Cook (1948), air being passed through at th rate of 1 litre per litre of medium per minute The material was harvested afte 5 to 7 days and yielded approximately the same quantity of crude active materia as was obtained by the stationary culture method, 1 e about 80 mg per litre

Extraction and concentration

The extraction and concentration process used was that of Abraham and Kent (unpublished, 1945)

" The liquid was brought to pH 10, then shaken with an equal volum of amyl acetate After settling, the amyl acetate was removed and shaken with three-tenths of its volume of acetate buffer at pH 4 The aqueou extract was brought to pH 10 and was again shaken three times with one third of its volume of amyl acetate which was then concentrated b distillation *in vacuo* to one-sixth of its volume This concentrate wa shaken three times with one-tenth of its volume of water, the pH of which was maintained at about 4 by the addition of HCl After concentration by vacuum distillation, the watery extract was dried by the lyophi process "

Separation of active substances

The counter-current distribution method developed by Craig and others was used to separate and characterize three antibacterially active substances from the crude product The procedure and theory of this method had been fully discussed in several papers (Craig, 1943, 1944, Craig, Golumbic, Mighton and Titus, 1945, Williamson and Craig, 1947, Barry, Sato and Craig, 1948), and they will not be considered here except in so far as required to make clear procedures which have been used and interpretations which have been made during the course of this work A total of 25 counter-current distributions were performed

The separation of a mixture of substances by the counter-current method depends on the relative distribution of the components between two immiscible liquids The advantages of using a system in which the substance to be investi gated is equally or nearly equally distributed between the two layers have been stressed by Craig and others A satisfactory partition of the active principles

of proactinomycin between diethyl ether and M/2 phosphate buffer was easily accomplished   In the early procedures a mixture of Na_2HPO_4 and KH_2PO_4 were used, but later K_2HPO_4 was substituted for the Na salt because the latter tended to crystallize

Eight tube distributions —These were carried out in 600 ml separating funnels, numbered 0 to 8   The two phases consisted of 200 ml quantities of mutually saturated ether and buffer, the lower layer being transferred   Equilibrium between the two phases was ensured by 100 inversions at each step   At the end of the distribution the solute in each funnel was completely transferred to the ether layer by adjusting the pH to 10 with 40 per cent NaOH and adding enough NaCl to give a saturated aqueous solution   The volume of each ether layer was then adjusted to 200 ml and transferred to a numbered bottle

Aliquots of the ether solutions were used for dry weight and activity determinations   For dry weight determinations one of two types of container was used, depending on the volume of the aliquot   For 1 or 2 ml aliquots, small aluminium cups weighing from 1 0 to 1 2 g were used   The lightest cup in the group was used as a counterpoise   For larger aliquots thin glass tubes were used, and with these the ether was evaporated by placing the tubes under a stream of air   The cups or tubes were then dried to constant weight *in vacuo* over P_2O_5 The total weight of the solute in the system was determined by totalling the dry weights per funnel and this was used in calculating the percentages shown on the graphs   The weighing error was estimated as $\pm$ 0 1 mg   The weight of the aliquot taken from the peak tube in most procedures exceeded the estimated error at least 40–50 times, and in one (Fig 6) 150 times

Assays of antibiotic activity were carried out by shaking an aliquot of each ether solution with M/15 phosphate buffer, pH 7 0, and evaporating the ether The buffer extracts were assayed by the cylinder-plate method and the percentage of the total activity in each determined by a comparison of the zones of inhibition with those of an aliquot of the starting solution, suitably diluted and assayed on the same plates   (In some cases, when only a rough determination of the activity was desired, filter paper discs cut with a cork borer were dipped in the ether solutions for the count of 5, dried by waving in the air for the count of 15, and placed on seeded plates (Sherwood, Falco, and de Beer, 1944))

Twenty-four tube procedures —Twenty-four tube distributions were carried out in an apparatus constructed at the Rockefeller Institute for Medical Research which is similar to that described by Craig (1944) except that it contains 25 tubes instead of the 20 of the earlier model   A small electric motor rotated the apparatus at the rate of 20 r p m , 50 revolutions (counted on a bicycle cyclometer) being used for each mixing, with a 5-minute period for the separation of the two layers before the next transfer

After completion of the 24 transfers the threaded metal plug in the top section of the apparatus was replaced by a cork carrying a short length of glass tubing By inverting the apparatus the contents of any tube could be emptied through this directly into a bottle with only a small loss of material   The organic solute in each tube was extracted into the ether layer by the addition of 40 per cent NaOH until the pH of the watery phase was 10   The layers were separated in a 20 ml separating funnel and the buffer shaken with fresh ether   The two ether extracts were combined and the volume made up to 15 ml   Dry weight and activity determinations were carried out as in the 8-tube procedure

RESULTS

Gardner and Chain (1942) suggested that should additional substan
similar origin to proactinomycin be discovered " this agent herein describe
be called Proactinomycin 1, the next Proactinomycin 2, and so on "
substances investigated in this paper are active components of this supp
single agent, they have been termed proactinomycin A, proactinomycin
proactinomycin C until their chemical structure is known   An inspect
preliminary results suggested that the substance with the highest pa
coefficient (hereafter called proactinomycin C or Pa C) might prove to be a
active substance and should be relatively easily separated   The separat
the components of the broad mixture in the centre of the curve was expec
offer some difficulties   The general plan adopted was to subject a sam
crude proactinomycin to an 8-tube funnel procedure and then to carry
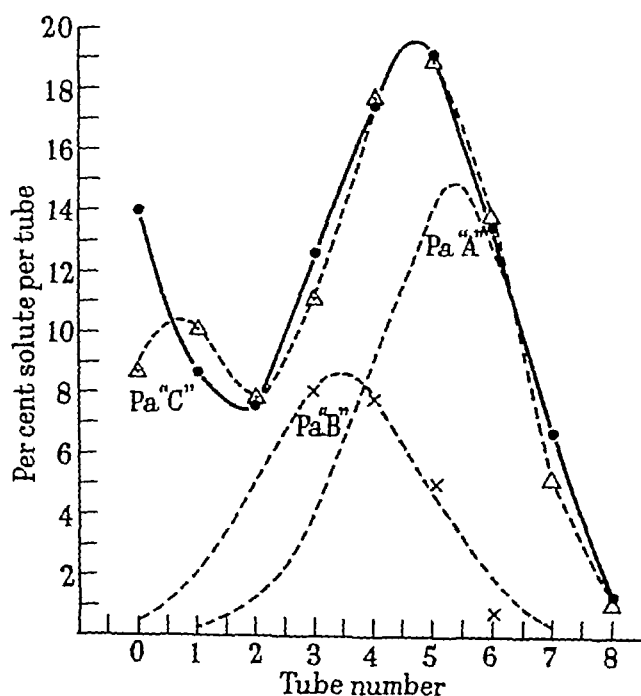
Fig 1 —(8 tube funnel distribution, lower phase moving)   pH of buffer, 7 0   The mate
used in this distribution was obtained from an aerated culture   The activity curve sho
that at least two active substances were present in the starting material as two peaks
present, i e at Tube 1 and Tube 5   Furthermore, the portion of the curve from Tubes
could only be explained by assuming the presence of at least two active substances in so
of these tubes   An attempt was therefore made to determine theoretical curves for t
substances which, when summed, would coincide with the dry weight curve over th
tubes

The theoretical curves were derived as follows   Curve A is a curve expanded from t
percentage of material in T_6 for a homogeneous substance having a K value of 0 55   The c
culated experimental K for T_6-T_7 is 0 534, and for T-T_8 is 0 575   Curve B is a theoreti
curve (K = 1 3) best fitting the points obtained by subtracting Curve A from the c
weight curve at T_3 to T_6 On the basis of these curves the starting material would have be
composed of 50 per cent of A and about 30 per cent of B

This interpretation is meant to be only approximate (note divergence in theoreti
and dry weight curves at T_4), but Fig 2 and 3 prove that it is very nearly correct

———●——— Experimental   - - - △ - - - - Activity
- - - - - - - - - Theoretical   ×   Experimental minus "A "

24-tube procedure on selected fractions rich in the substance having the lowest partition coefficient (termed practinomycin A or Pa A)   A second 24-tube procedure would then be carried out on a mixture of substance Pa C and the substance proactinomycin B (or Pa B), having a partition coefficient intermediate between Pa A and Pa C   Fig 1, 2 and 3 show the results of this approach on a sample of powder obtained from an aerated culture   It will be noted that the pH of the buffer in each procedure was chosen so that the best separation might be gained   In general, with the crude material the pH was 7 0, with Pa A 7 2, with Pa B or mixtures of Pa B and Pa C 6 8, and with Pa C alone 6 5   The results shown in Fig 1, 2 and 3 were encouraging, but other batches of material were not so satisfactory as they contained a larger percentage of inactive impurities ,

one of these was especially difficult to deal with as it exhibited a partition coefficient between that of Pa B and Pa C   The reason for this difference between different batches is not definitely known

The graphs given in Fig 1 to 6 have been selected from the 25 runs made to illustrate most clearly the kind of results obtained and the procedure adopted in certain cases

Isolation of Proactinomycin A, B and C

Proactinomycin A —As already stated (Fig 3), homogeneous or nearly homogeneous Pa A could be crystallized readily from a concentrated wet ether solution The crystals formed as short, thick plates or short prisms   Recrystallization attempts with any of the solvent mixtures tried (ether or 40°–60° petroleum ether with chloroform, alcohol or acetone) were unsuccessful   However, crystals with a melting point of 168–169° C (from T_9, Fig 6) could be obtained from crystals contaminated with a small amount of impurity by first washing with ether, then dissolving the crystals in a small quantity of chloroform and adding ether to the chloroform solution   After melting point determinations, crystals of Pa A recrystallized on cooling   Attempts to crystallize Pa A from very impure solutions were not successful
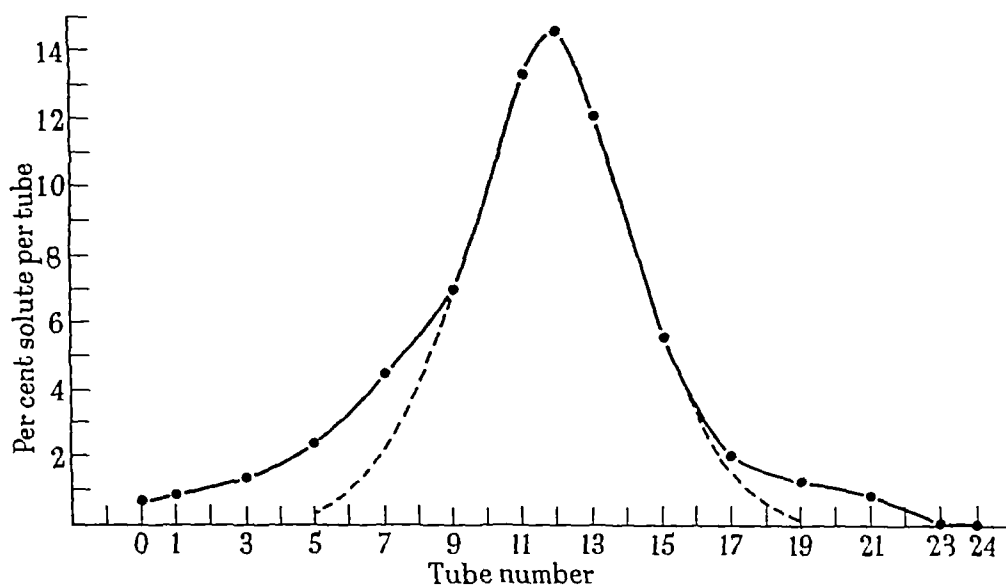
FIG 3 —(24 tube machine distribution)   pH of buffer, 7 2   The material used in this procedure was obtained from T_6, T_7 and T_8 of Fig 1, to which was added a small amount of other material rich in Pa A from previous distributions   The ether solution was concentrated and the water removed as described under Fig 2   This concentrated solution was left in the refrigerator overnight   Next morning many " button like " groups of crystals (M P 165–167° C) were present on the sides of the vessel   The mother liquor gave the distribution shown in this curve

An aliquot of the ether solution from T_{11}, evaporated quickly under reduced pressure, yielded a clear, colourless, sticky oil which hardened on drying over P_2O_5 When a small amount of ether was added to the tube containing this oil, the fluid immediately became turbid, and in a few minutes crystals could be detected   These crystals were soluble in chloroform, acetone, alcohol and dilute acid, only slightly soluble in ether and light petroleum ether, and insoluble in water and dilute alkali   The melting point on the hot stage micro melting point apparatus was 168–169° C

———●——— Experimental   - - - · - - - - - Theoretical

Proactinomycin B —A doubtfully crystalline solid could be obtained from pure Pa B by adding light petroleum ether to the homogeneous free base obtained as an oil after evaporation of an ether solution   These " crystals " melted at 83–87° C on the hot stage micro-melting point apparatus

Proactinomycin C —Attempts to crystallize this substance were not successful
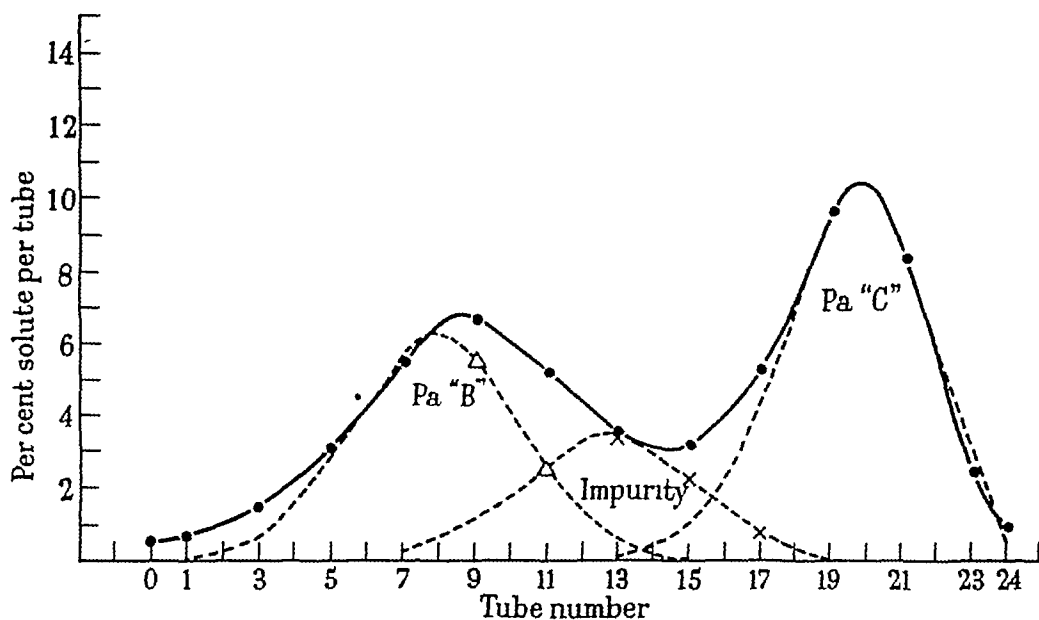
FIG 4 —(24 tube machine distribution)   pH of buffer, 6 8   This curve is presented to show the presence of a homogeneous inactive substance with a partition coefficient between that of Pa B and Pa C   The inconvenience which this impurity caused from a preparative standpoint, when present in relatively large amounts, is seen from a comparison of this curve with Fig 2   In the latter at least four tubes containing the largest amount of Pa B were selected as being homogeneous within the limits of the method, whereas in the present case, none of the tubes were used as a source of material for experiments on Pa B until additional distributions had been performed

The yield of homogeneous Pa C was also markedly reduced by the presence of this impurity

———●——— Experimental   ✕ Experimental minus " C "
· · · · · — · · — · · · Theoretical   △ Experimental minus " Impurity "
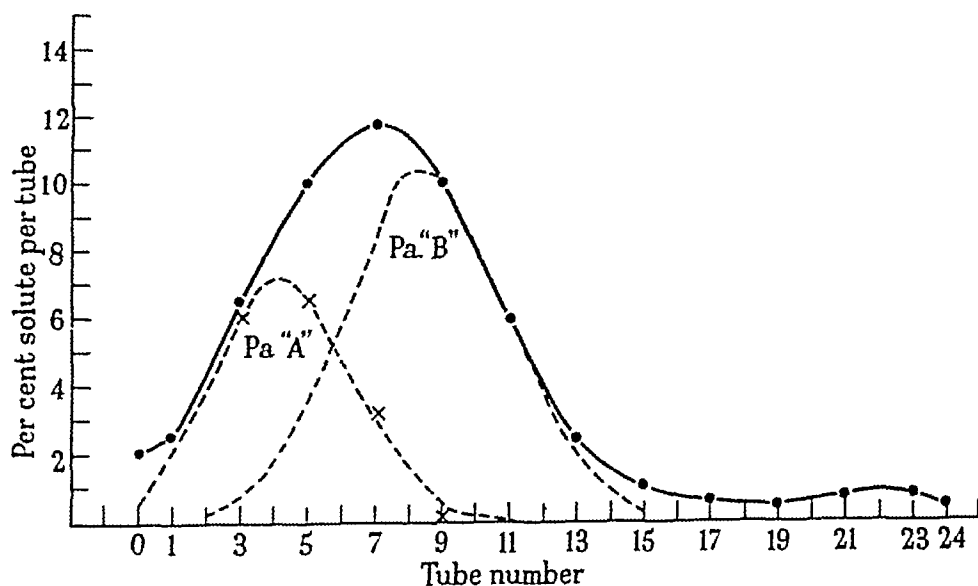
FIG 5 —(24-tube machine distribution)   pH of buffer, 6 8   This curve shows that when Pa A and Pa B were both present, little separation was obtained in 24 transfers K for Pa A = 0 23, and for Pa B = 0·56   The starting material was obtained from selected tubes from a 16 tube alternate withdrawal procedure (Craig, Hogeboom, Carpenter and du Vigneaud, 1947) in funnels

———●——— Experimental   · · · · · · · · · · Theoretical   ⋎ Experimental minus " B "
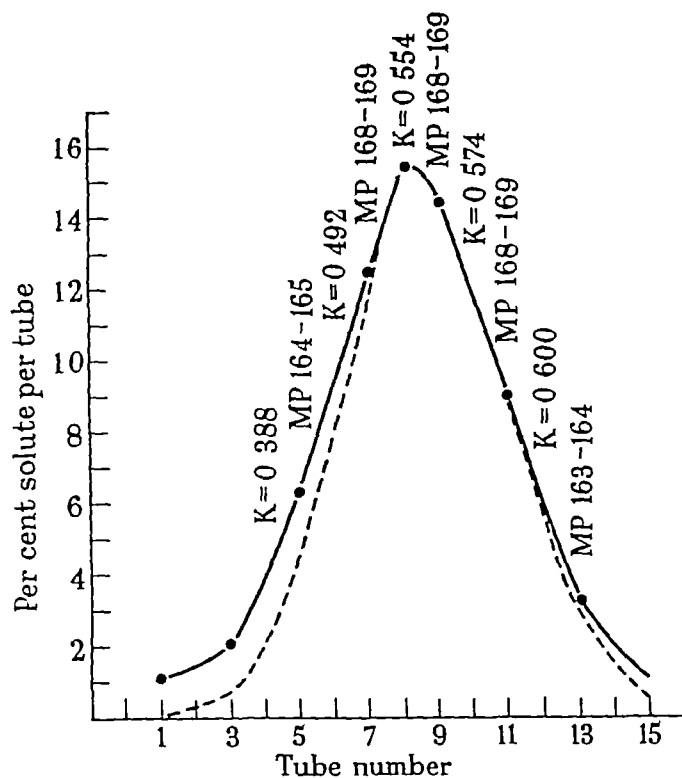
FIG 6 —(24 tube machine distribution)   pH of buffer, 7 2   This distribution was carried out on crystals of Pa A   Ten ml aliquots were used for dry weight determinations and the melting points of crystals from five tubes were determined

———•——— Experimental   - - - - - - - - - Theoretical

Chemical properties of proactinomycin A, B and C

Chemical analyses (by Weiler and Strauss) of the three antibiotics gave the following results in percentages

	Proactinomycin A	Proactinomycin B	Proactinomycin C
C	63 2	63 8	65 5
H	9 04	9 4	9 2
N	2 67	2 71	3 08
O (by difference)	25 09	24 09	22 22
(N)CH$_3$	7 35	8 7	8 5
(C)CH$_3$	18 8	18 04	18 6
OCH$_3$	1 1	0	0
Molecular weight (Rast)	445	513	322

(The sulphur content of the three samples was 0 0096, 0 041 and 0 057 per cent respectively)

On the basis of these figures the following molecular formulae are suggested
  Proactinomycin A, $C_{27}H_{47}O_8N$ (Requires C, 63 2 , H, 9 2 , N, 2 7)
  Proactinomycin B, $C_{28}H_{49}O_8N$ (Requires C, 63 8 , H, 9 3 , N, 2 7)
  Proactinomycin C, $C_{24}H_{41}O_6N$ (Requires C, 65 6 , H, 9 3 , N, 3 2)

The analytical figures indicate that proactinomycin A and proactinomycin B are closely related compounds   The values for (N)CH$_3$ show that all three

antibiotics contain an $-$ N(CH$_3$)$_2$ grouping   This is compatible with the finding of Philpot (unpublished, 1946) that dimethylamine was a product of the treatment of crude proactinomycin with alkali

Ultraviolet spectra

The ultraviolet absorption curves given by solutions of Pa A, Pa B and Pa C in anaesthetic ether are shown in Fig 7   Measurements were made with a Beckman model DU photoelectric spectrophotometer and absorption due to solvent alone was automatically subtracted   The cell length was 1 cm   The measurements were made by Dr V E Sharp and Dr G V R Born
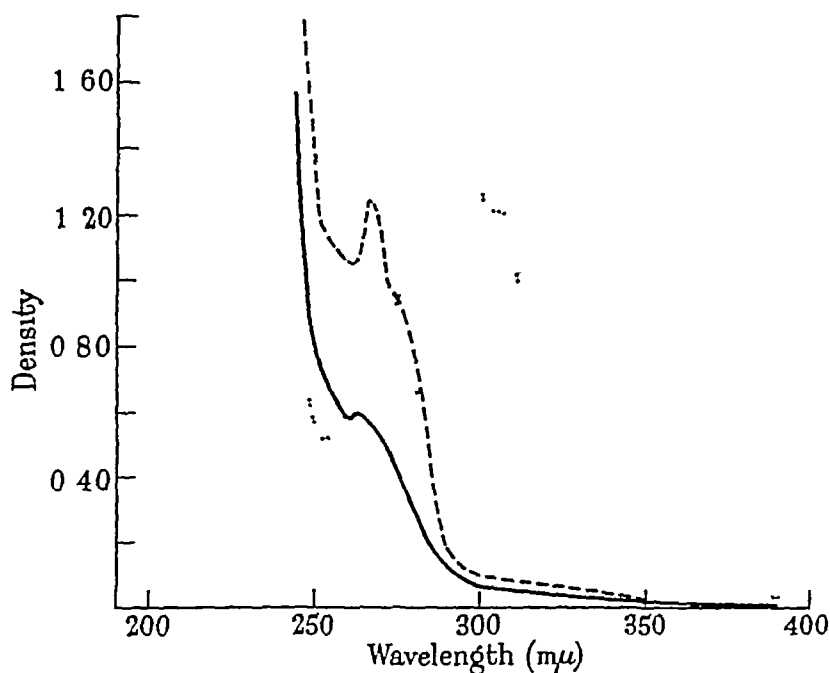
Fig 7 —Ultraviolet absorption of proactinomycin A, B and C   Measured in the Beckman model DU spectrophotometer in anaesthetic ether   Thickness of layer 1 cm

—————————— Pa "A" 0 5 mg /c c
-    -   - - Pa " B " 0 5 mg /c c
               Pa " C " 0 01 mg /c c

SUMMARY

A reinvestigation of proactinomycin has resulted in the isolation of three basic antibiotics, one of which has been obtained in a crystalline form

The work reported in this paper was carried out under the general supervision of Dr N G Heatley   Mr G G F Newton has offered many helpful suggestions during the course of the work, and invaluable technical assistance was given by Mr J Kent and Miss S Long

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THE ANTIBACTERIAL AND PHARMACOLOGICAL PROPERTIES OF THE PROACTINOMYCINS A, B AND C

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THE separation of three distinct proactinomycins from the antibacterial products of *Proactinomyces* (*Nocardia*) *gardneri* has been described (Marston, 1949) The present paper describes investigations of some of the antibacterial and pharmacological properties of these three substances, Proactinomycins A, B and C (called for convenience Pa A, Pa B and Pa C), and of the unfractionated substance, Pa crude

EXPERIMENTAL

Antibacterial Properties

Methods

The antibacterial titres of the preparations were ascertained by the serial dilution method Two-fold serial dilutions in distilled water, pH 6 5, were sterilized by filtration through a sintered glass filter, and were kept in the refrigerator when not being used The same batch of heart extract broth was used for all tests, but 10 per cent serum was added for *Str pyogenes*, *Str pneumoniae* and *C diphtheriae*, and 2 per cent glucose in addition for *N meningitidis* Each tube containing 1 8 ml of medium and 0 2 ml of antibiotic solution was inoculated with one drop (delivered with a Dreyer's pipette) of an overnight culture of the organism (2 drops of *N meningitidis*)

The tests were read after 24 and 48 hours of incubation at 37° C , but for the sake of clarity only the 48-hour results are given in the tables In general the 24-hour readings showed inhibitory titres 2 to 4 times as high as those at 48 hours

To ascertain the effect of serum on antibacterial activity 10 per cent of sterile horse serum was added to the medium in serial dilution tests To ascertain whether whole blood impaired activity, the following procedure was adopted

Five ml of each preparation were made isotonic to horse red blood cells by the addition of 0 5 ml of 9 per cent sodium chloride To each of these solutions was added 4 5 ml of oxalated whole blood, and the mixtures were rotated on a

tilted gramophone turntable (Heatley, 1941) for 4 hours at 37° C   They were then centrifuged and the clear supernatants, which were coloured by haemolyzed blood, were removed to four separate tubes which were placed in the refrigerator overnight and tested the following day by the serial dilution method against *Staph aureus*

Ten ml of distilled water were added to the red cell pack (approximately 1 ml in volume) remaining in each tube   This procedure readily lysed the cells, and these solutions were tested by the same method

RESULTS

Activity against various bacteria

The results of the serial dilution tests are given in Table I   Three of the preparations had about the same activity, but Pa B was distinctly inferior against all the organisms tested except *N meningitidis*

In view of Abraham and Duthie's investigation (1946) of the effect of pH on the antibacterial activity of proactinomycin, the pH of the medium was determined at the end of two of the tests (*B anthracis* and *Staph aureus*)   There was no difference in comparable tubes containing different preparations The apparent inferiority of Pa B is not, therefore, to be explained by a change in pH

TABLE I —*Comparison of the Antibacterial Activity of the Four Preparations of Proactinomycin against Various Bacteria by the Serial Dilution Method Figures show the Dilution of the Substance at the End-point indicated*

Preparation	Kind of inhibition	Staph aureus	Str pyogenes	Str pneumoniae
Pa crude	Partial	1 40,000	1 640,000	1 1,280,000
	Complete	1 20,000	1 160,000	1 640,000
Pa A	Partial	1 40,000	1 640,000	1 1,280,000
	Complete	1 20,000	1 80,000	1 640,000
Pa B	Partial	1 10,000	1 160,000	1 640,000
	Complete	—	1 20,000	1 160,000
Pa C	Partial	1 80,000	1 640,000	1 1,280,000
	Complete	1 20,000	1 80,000	1 640,000

Preparation	Kind of inhibition	B anthracis	C diphtheriae var gravis	N meningitidis
Pa crude	Partial		1 640,000	
	Complete	1 80,000	1 160,000	1 80,000
Pa A	Partial	1 80,000	1 320,000	
	Complete	1 40,000	1 160,000	1 80,000
Pa B	Partial	1 20,000	1 80,000	
	Complete	—	1 40,000	1 160,000
Pa C	Partial		1 1,280,000	
	Complete	1 80,000	1 320,000	1 40,000

A dash indicates that the figure was outside the range of the titration

A blank indicates that there was no partial inhibition, as tubes showing no growth and full growth were adjacent in the dilution series

The *B anthracis* and *C diphtheriae* var *gravis* were laboratory stock strains   The other organisms were as follows *Staph aureus*, Wellcome CN 491 , *Str pyogenes*, Wellcome CN 10 , *Str pneumoniae*, Wellcome CN 33 , and *N meningitidis*, Colindale II 1615

Effect of serum

The presence of 10 per cent horse serum had no marked effect on the anti-bacterial activity of any of the four preparations against *Staph aureus* (Wellcome CN 491)

Effect of whole blood

Florey, Jennings and Sanders (1945) found that the activity of proactinomycin was reduced by incubation for 4 hours with undiluted whole blood When this was repeated by the method described above there was an indication of slight loss of activity by all the substances, but residual activity was in all cases at least 50 per cent   Separate titration of the red cells after lysis showed an approximately equal distribution of the antibiotics between the red cells and the surrounding fluid

Effect of size of inoculum

The titre of all four preparations against *Staph aureus* was only moderately affected by changes in the size of the inoculum   In each case a thousand-fold reduction in the density of the inoculum resulted in an increase in titre, usually about four-fold (Table II)

TABLE II —*Effect of Inoculum Size on the Antibacterial Activity of the Four Preparations against* Staph aureus   *Figures show the Dilution of Substance Completely Inhibiting Growth*

Preparation	Inoculum overnight culture of *Staph aureus*		
	Undiluted	Diluted 1 1000	Diluted 1 1,000,000
Pa crude	1 20,000	1 80,000	1 640,000
Pa A	1 20,000	1 80,000	1 160,000
Pa B	—	1 20,000	1 40,000
Pa C	1 20,000	1 80,000	1 320,000

Relation of titre by dilution test to zone size in cylinder-plate assays

Under the conditions used (cylinder-plates surface seeded with a 1 100 dilution of a broth culture of *Staph aureus*) a solution containing about 0 2 mg /ml (1 in 5000) of any of the preparations gave a zone of inhibition about 20 mm in diameter   The concentration-zone diameter curve obtained by the cylinder-plate assay was fairly steep, the zone diameter increasing by approximately 2 mm for each doubling of concentration

Pharmacological Properties

Acute toxicity to mice

One hundred and ten 20 g mice in groups of from 5 to 8 were injected intra-venously with various amounts of substances Pa A, Pa B, Pa C and Pa crude dissolved in 0 3 ml of saline   The approximate LD_{50} of each substance was calculated by the method of Reed and Muench (1938), and was found to be for Pa A 3 0 mg , for Pa B 2 4 mg , for Pa C 1 6 mg , and for Pa crude 1 8 mg

Some reaction was noted in all of the mice tested except those receiving

Pa A 1 8 mg and Pa crude 1 3 mg   Symptoms began immediately after the injections, and ranged from a slightly shaky gait to violent convulsions followed by death in from 1 to 5 minutes   In some cases cyanosis was present   All of the mice which survived appeared normal 10 to 15 minutes after the injection, and from that time to the end of a 10-day observation period they presented no abnormalities

Toxicity to leucocytes in vitro

Effect on motility —Tests in which an antibacterial substance is brought into direct contact with leucocytes *in vitro* give an opportunity for direct observation of the effect of the substance on living cells, and are also therapeutically important because of the essential role played by leucocytes in complementing the curative action of bacteriostatic substances

The effects of the three substances Pa A, Pa B and Pa C on the motility of human leucocytes *in vitro* were compared by the method described by Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941)   Each substance was tested in 9 dilutions ranging from 1 to 500 to 1 to 80,000   Some of the dilutions were tested more than once, and these duplicate tests gave consistent results   Pa A, which was the least toxic, killed leucocytes in 20 minutes at a concentration of 1 in 500, and impaired their motility at 1 in 10,000, but a concentration of 1 in 20,000 was without effect during an hour's observation   Pa B at 1 in 500 killed them more quickly, and at 1 in 25,000 impaired motility after an hour   Pa C was considerably more toxic than either of the others, as a concentration of 1 in 8000 killed the cells in 20 minutes, and 1 in 40,000 killed them in an hour

Effect on phagocytosis —Leucocytes were obtained from the peritoneal cavity of rabbits, after the injection of saline on two successive days, by the method described by Mudd, Lucké, McCutcheon and Strumia (1929)   These were brought into contact with heat-killed staphylococci suspended in 5 per cent serum in saline containing different concentrations of the substances, by slow rotation at 37° C for one hour   At the end of this time smears were made and fixed, and stained by Claudius' modification of Gram's stain, and the number of cells containing bacteria was counted

There was no appreciable   nce between the number of cells containing bacteria (about 80 r   preparations and in preparations containing concentration   Partial   h as 1 2000   Phagocytosis in concentrations of   Complete   about the same, but in 1   2000 of this subst   Partial   ingested bacteria

Thu   Complete   than the other two substances to leucocytes   Partial

Effect o   mplete   the substances to tissue cells was further teste   tial   al space of rabbits, thus bringing them into dire   ct wit   rous system   The procedure was as follows   The rabbits were   he preoperatively and anaesthetized with ether   The spinal cord   by a cisternal puncture, and 0 5 ml of cerebro-spinal fluid withdr   ced by the same quantity of a saline solution (pH 6 5) containing fi   ng of the antibiotics   After recovery from the anaesthetic the animal   hed for signs of irritation of the central

nervous system In most cases they were killed 24 hours after the injection, when a sample of the cerebro-spinal fluid was assayed for antibacterial activity and the brain stem was inspected

In only one rabbit (after injection of 2 mg of Pa B) was any gross damage to the nervous system, presumably due to the needle, found in the post-mortem examination In this case haemorrhage had extended into the substance of the brain stem

No symptoms which could be related to the effect of substances Pa B and Pa C were noted from maximum doses of 6 and 12 mg respectively, but Pa A obviously had a detrimental effect when injected into the spinal canal After-effects varied from post-operative " head shaking " when 3 mg were injected, to death within 5 to 10 minutes after the injection of 10 mg Six mg in one rabbit caused marked symptoms, which began while the animal was still recovering from the anaesthetic, and consisted of, first, vigorous shaking of the head, then scratching motions and inability to stand About 45 minutes after the injection it began to have fits every two or three minutes These started with rapid, side-to-side motions of the head, and progressed quickly to violent, unco-ordinated jerking of the whole body with a tendency to fall to the right

The frequency and violence of the fits decreased rapidly during the next 30 minutes, and four or five hours after the injection the rabbit appeared quiet but otherwise fairly normal After the seizures had become less frequent and less violent, stroking the head tended to bring on involuntary shaking of the head, but noise had no effect

Symptoms similar to these, but less violent, were noted in one out of two rabbits given 10 mg of Pa crude, but not in the other

Antibacterial activity was detected in the cerebro-spinal fluid 24 hours after injection in animals that received as much as 3 mg of Pa A, 2 mg of Pa B, and 12 mg of Pa C

The most interesting feature of these results is that the order of toxicity to nerve tissue, as shown by these tests, differed from that to whole mice and to leucocytes *in vitro* Pa A was the most toxic of the three substances to the central nervous system, and was the least toxic of the three in the other tests

Effect on blood pressure and respiration of decerebrate cats

Florey, Jennings and Sanders (1945) found that proactinomycin caused a rise in the blood pressure of cats under chloralose anaesthesia A rise was obtained under nembutal anaesthesia, though on repeating the injection of the antibiotic a fall occurred In the decerebrate cat there was a slow rise of pressure after an initial fall, while in the decapitated cat injection was followed by a sharp fall, with recovery up to but not above the original level

In the present experiments the effects of Pa A, Pa B, Pa C and Pa crude on the blood pressure were investigated on six decerebrate cats, and Pa crude on one cat under chloralose anaesthesia The method of decerebration was that described by Schmidt (1923), in which the brain is divided by drawing a ligature tight, so that bleeding is avoided

The substances were dissolved in 5 to 8 ml of saline, pH 6 5–7 0 and were administered slowly taking two or three minutes, by intravenous injection The results obtained in these experiments with Pa crude resembled those reported by Florey *et al* with proactinomycin in the decerebrate cat in that the pressure

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FIG 1 —*Cat* м/4 (decerebrate)   Weight 2 8 kg (11 minute break between end of first and beginning of second tracing)   The injection of 100 mg of Pa A (36 mg per kg) caused a slight fall of blood pressure (10 mm of Hg) followed by a slight rise   The respiratory rate decreased from 21 to 15 r p m during the injection   Thirty minutes after this injection 100 mg of Pa crude (36 mg per kg) were injected into the same cat   This dose caused a pronounced fall in blood pressure, from 118 mm to 40 mm, of mercury, and an increase in respiratory rate from 32 to 43 r p m   [The time signal in Fig 1 to 5 indicates 10 seconds]

FIG 2 —*Cat* м/3 (decerebrate)   Weight 2 6 kg (10 minute break between end of first and beginning of second tracing)   An injection of 50 mg of Pa B (19 mg per kg) had no immediate effect on the blood pressure, but a slight rise occurred, starting a few minutes after the injection was completed   There was no effect on the rate of respiration   Fifty mg of Pa crude were injected 20 minutes after the first injection, and resulted in the marked fall in blood pressure and increased respiratory rate shown in the adjacent tracing

fell and then rose again   In the present work, however, the initial fall was more pronounced, and though there was afterwards recovery, the original level was only exceeded in a few animals   In the one cat tested under chloralose
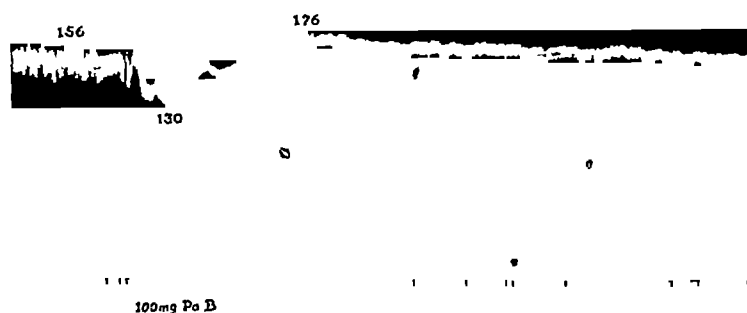
FIG 3 —*Cat* M/7 (decerebrate)   Weight, 2 8 kg   One hundred mg of Pa B, or 36 mg per kg caused a fall in blood pressure followed by the most pronounced rise (20 mm of mercury) encountered in these experiments

FIG 4 —*Cat* M/5 (decerebrate)   Weight 2 8 kg   An injection of 50 mg of Pa C (18 mg per kg) caused a fall in blood pressure of over 100 mm of mercury and doubled the respiratory rate   Forty five minutes after this injection 50 mg of Pa crude (18 mg per kg) were injected and caused the same type but much less marked changes in blood pressure and respiration

with Pa crude the result differed from those of Florey *et al* in that the pressure fell steeply before recovering and eventually showing a rise above the original level

Fig 1 2 3 4 and 5 illustrate the results obtained with Pa A, Pa B, Pa C and Pa crude from which it would appear that Pa C is largely responsible for the fall of blood pressure associated with the injection of Pa crude

Effect on isolated smooth muscle

In these experiments one horn of the uterus of a guinea-pig was suspended in 50 ml of oxygenated Ringer s solution in a container immersed in a water bath, the temperature of which was maintained at 37° C   The substances to be tested were dissolved in Ringer s solution and injected through a glass tube, the opening of which was just above the oxygen inlet and below the suspended segment of uterus
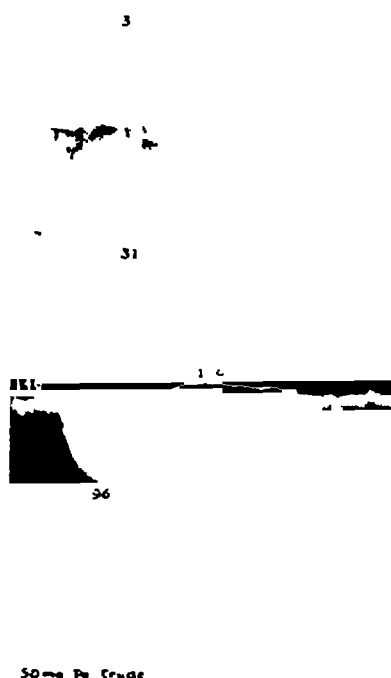
Fig 5 —Cat ♂/2 (decerebrate)   Weight 2 4 kg   An injection of 50 mg of Pa crude (21 mg per kg) caused a pronounced fall in blood pressure followed by a slight rise above the original level and an increase in respiratory rate and depth

Fig 6 7 8 and 9 show the type of response obtained in each case   The dilutions given in the tracings are the final dilutions present in the fluid surrounding the muscle   The time marker interval in all figures = 30 seconds

Effect of Pa crude

Fig 6 shows the type of response obtained with a concentration of 1/10,000 of Pa crude

A concentration of 1 in 25 000 depressed the amplitude of uterine contractions, and a concentration of 1 in 5000 caused them to cease almost entirely   In both cases recovery occurred after washing with warm Ringer s solution
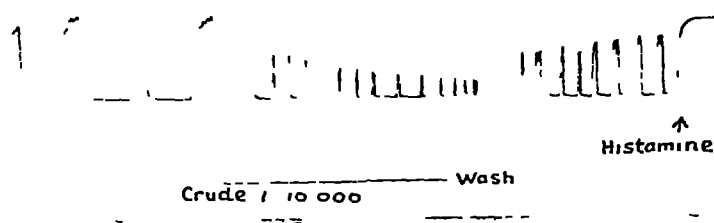
Fig 6

Effect of Pa A

Fig 7 shows the type of response obtained with Pa A at a concentration of 1 in 10,000   There was a slight decrease of amplitude of the contractions and increase of their frequency   A concentration of 1 in 25,000 had no noticeable effect on the uterine contractions
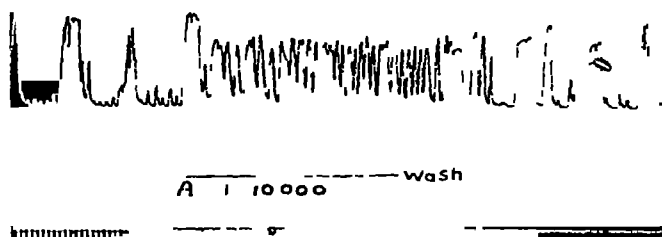
Fig 7

Effect of Pa B

The type of response following contact with Pa B is shown in Fig 8   A concentration of 1 in 10,000 of Pa B caused a marked contraction of the uterus, which was maintained until the uterus was washed with fresh Ringer's solution, after which the original rhythm returned
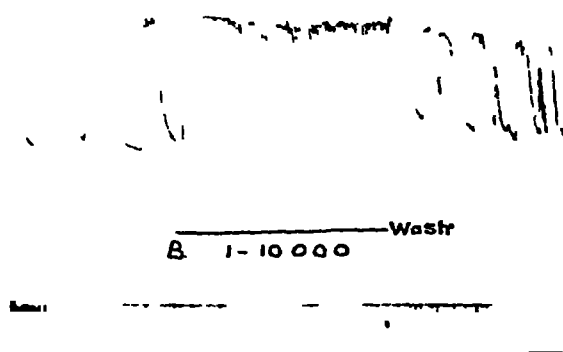
Fig 8

A concentration of 1 in 25,000 did not significantly alter the amplitude or frequency of contractions, but when this same muscle was subjected to a concentration of 1 in 25,000 of Pa C the contractions practically ceased

Effect of Pa C

Fig 9 shows the effect of Pa C at a concentration of 1 in 12,500 A concentration of 1 in 100,000 caused an appreciable decrease in the rate of uterine contractions, whereas a concentration of 1 in 50,000 practically abolished the contractions However, even after being subjected to a concentration of 1 in 12,500 for 20 minutes, rhythmic contractions were restored after washing with Ringer's solution
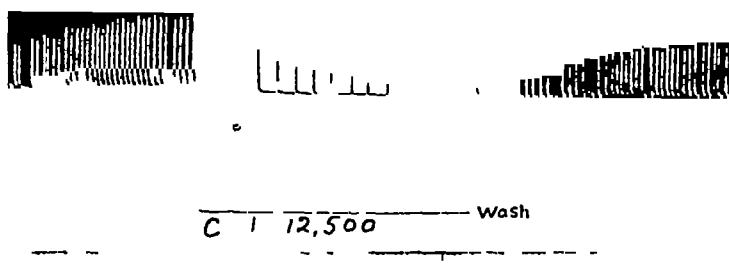
FIG 9

It is thus clear that the inhibition of the rhythmical contractions of the guinea-pig uterus by Pa crude can be attributed to its content of Pa C, as both Pa A and Pa B stimulate rather than inhibit contraction

Excretion in Mice

Methods

Two mg dissolved in 0 5 ml of saline were administered by stomach-tube to each of three mice Pa A, Pa B and Pa C were each given in this way Two mg of Pa A in 0 5 ml of saline were also injected subcutaneously into each of three 20 g mice

After 5 hours the mice were killed and the urine collected and assayed for antibacterial activity on cylinder-plates seeded with *C xerosis* The plates also carried solutions of known strength in terms of which recovery was calculated The gastro-intestinal tract was removed from mice that had received substance orally and rinsed with 2 ml of saline These washings were also assayed

Results

The figures given below which show the amount of substance recovered, are the averages of determinations on each group of three mice The errors in their estimation are probably large

Substance	Route	Percentage recovered in	
		Urine	Gastro intestinal washings
Pa A	Oral	20	3
Pa B	,,	20	$1\frac{1}{2}$
Pa C	,,	$2\frac{1}{2}$	3
Pa crude	Subcutaneous	20	—

Detection in the blood after subcutaneous injection in mice

The blood was collected from the heart of mice that received Pa A subcutaneously in the previous experiment when the animals were killed   It exhibited no inhibition (1) against *Staph aureus* when tested by the capillary tube method (Fleming, 1944), or (2) against *C xerosis* when tested by the cylinder-plate method   The lowest concentrations detectable by these methods were approximately 1   40,000 by method (1), and 1   160,000 by method (2)

In another experiment each of three 18 g   mice was given 2 mg   of Pa A subcutaneously   One mouse was killed 15 minutes after the injection, and the others 30 and 60 minutes after injection   Samples of heart blood were assayed by methods (1) and (2) above   No definite activity was detected in any of the samples, but there was a small, questionable zone of inhibition on the *C xerosis* plate around the cylinder of the 1-hour sample

Protection Tests in Mice

Owing to the smallness of the supplies available, only one protection test could be carried out with Pa A   For this the pneumococcus was chosen, as it was the most sensitive of the bacteria that had been tested *in vitro*, and a large dose of organisms was not needed to kill mice   Six groups of 10 mice weighing about 20 g   were infected intraperitoneally with 0 2 ml   of a 1 in 10,000 dilution of an 8-hour culture of *Str pneumoniae* (Wellcome CN 33)   Thirty minutes after infection the first dose of Pa A was injected subcutaneously   The injections were repeated every three hours for 48 hours   Table III shows that the largest dose of Pa A given subcutaneously afforded some slight protection, and probably better protection could have been obtained by a larger dose   But the margin between toxic and protective doses is evidently small

TABLE III

Single dose, mg	Route	Number of mice surviving at the end of hours								
		18	24	30	36	42	45	57	63	69
0 1	Subcutaneous	10	7	3	2	0			..	
0 2	,,	10	10	4	2	1	0			
0 4	,,	10	10	9	4	2	0			
0 8	,,	10	10	10	10	10	7	2	1	0
0 1	Oral	10	4	0						
	(Control)	10	8	3	1	0				

SUMMARY

Proactinomycin A and Proactinomycin C had approximately the same antibacterial activity against the organisms tested, while Proactinomycin B was about half as active as the other two

The substances were shown to be relatively toxic when injected intravenously into mice, and in sufficient concentrations to decrease the motility of human leucocytes, to depress the blood pressure of decerebrate cats and to alter the normal contractions of the guinea-pig uterus   In all of these tests Proactinomycin C was found to have the most pronounced and Proactinomycin A the least pronounced effect   Similarly Proactinomycin C was the only one of the three substances that decreased phagocytosis of bacteria by leucocytes in the concentrations used   Proactinomycin A caused severe disturbance when injected in sufficient quantity into the cerebro-spinal fluid of rabbits, whereas the same amount of Proactinomycin B or Proactinomycin C had no noticeable effect

All three substances were detected in the urine and gastro-intestinal washings of mice after oral administration, and Proactinomycin A (the only one tried) was detected in the urine after subcutaneous injection

In the doses used, Proactinomycin A offered little protection to mice infected with *Str pneumoniae*   Larger doses would have approached the toxic level

In view of the low antibacterial power relative to toxicity, it is unlikely that any of the three substances investigated would be useful clinically as antibacterial agents

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THE GENETIC CHARACTER OF O-D CHANGE IN INFLUENZA A

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IN 1943 Burnet and Bull showed that fluids obtained from initial isolations of influenza A virus by the method of amniotic inoculation gave unusual haemagglutination reactions Fowl cells were not agglutinated or showed a very low titre of incomplete agglutination, but human or guinea-pig cells were agglutinated to a titre in conformity with the infectivity of the fluid By transfer in the amniotic cavity at limiting dilutions it is possible to maintain the original, O phase, character One of the 1942 strains, BEI, is still available in this laboratory in the O form Without these precautions passage by any method in the chick embryo speedily results in the appearance of the D phase, agglutinating fowl cells to the same titre as human or guinea-pig cells

In all the isolations of influenza A that have been made in Melbourne since 1942 virus was first obtained in the O phase Workers in other laboratories have for the most part confirmed this finding (Dudgeon, Mellanby, Glover and Andrewes, 1948) A number of isolations have, however, been reported by the use of allantoic inoculation, with recognition of the virus by fowl cell agglutination (Rickard, Thigpen and Crowley, 1944) Nevertheless, all are agreed that allantoic inoculation is far less effective than inoculation of 13-day embryos in the amniotic cavity, and an interpretation of the significance of the results of Rickard *et al* has already been published (Burnet and Stone, 1945a)

There has been no confirmation from other laboratories of maintenance of a strain indefinitely in the O phase by passage at limiting dilutions This is necessarily a time-consuming and difficult task not likely to be undertaken by anyone not deeply interested in the specific problems The D phase or an intermediate always appears at each passage and correct dilutions and appropriate timing of harvesting are needed to maintain the O character In the light of our experience, a strain whose characters can be readily maintained by chick embryo passage is not in the O phase

The stimulus to publish the work reported in this paper was the appearance of Magill and Sugg's (1948) paper in which it is claimed that the O–D change has no genetic significance, but represents merely a change which can be produced at will by altering the ionic environment of the virus Briody (1948), in this laboratory, had previously shown that by simple heating, some strains with O type of agglutination reactions could be converted into material agglutinating fowl cells

It seemed to us desirable that we should describe our own studies of this type on various O and intermediate phase strains of influenza A It will be shown

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that influenza A virus can exist in a form which maintains its O character despite the application of any of these methods   It can be converted to the D only by the occurrence of an appropriate mutation in the course of multiplication in chick embryo tissues   It is only intermediate forms which can be modified *in vitro* to give O or D reactions

We would point out that in our original paper O phase is ascribed to many strains which we should now regard as intermediates   In a more recent paper (Anderson and Burnet 1947) a strain IAN–O was described which was said to be capable of growing in the O-phase in the allantoic cavity   This interpretation will be shown to be wrong   The strain is in fact a particularly good example of the intermediate type of virus which allows the various changes described by Magill and Sugg to be demonstrated

MATERIALS AND METHODS

Viruses

BEL —Influenza A strain isolated during Victorian epidemic 1942

O and intermediate (false O) phase virus was obtained by amniotic inoculation of material which had been maintained in the O phase during 26 amniotic passages at limiting dilutions (method of Burnet and Stone 1945*b*)

D phase virus had received numerous allantoic passages

IAN —Influenza A strain isolated in 1946 from a sporadic case of influenza in Melbourne   The virus used was the substrain previously described as in the *O phase*   It was prepared by allantoic inoculation of material which had received 6 allantoic passages since its isolation in the amniotic cavity   Its properties are described by Anderson and Burnet (1947)

Oc I —Influenza A strain isolated from an epidemic in Ocean Island 1948

O and intermediate (false O and δ) phase virus was obtained during the first or second amniotic passage of the strain

The following strains were propagated in the allantoic cavity

WSE —Egg-adapted substrain of the classical WS (influenza A) strain

MEL —Melbourne strain of influenza A

LEE —Classical strain of influenza B

SW —Shope s strain 15 of swine influenza virus

NDV —Newcastle disease virus isolated by Albiston and Gorrie 1931

Haemagglutination titrations —Equal volumes (0 25 ml) of virus dilutions and 1 per cent red cells were mixed and allowed to settle   Readings were made by observing the pattern of sedimented cells as described by Burnet (1942)   Partial (÷) agglutination was taken as the end-point   Tests were carried out at room temperature or at 4° C as specified in the text   Comparative titrations were made with fowl and guinea-pig cells and the titres are expressed as ratio of the two end-points F/G

Amniotic inoculation —The techniques have been described in previous communications from this laboratory   After inoculation the eggs were incubated for 40–42 hours at 35° C   Samples of amniotic fluid were then withdrawn at regular intervals until fluids were obtained which agglutinated human cells the amniotic fluids were then harvested   The lungs were removed ground with alundum suspended in 1 ml normal (0 9 per cent) saline and lightly centrifuged   The supernatants were used as lung emulsions

Receptor-destroying enzyme of V cholerae (RDE) was prepared by the method of Burnet and Stone (1947)

Character of Variants of a Recently Isolated Strain

The characteristics of an epidemic of influenza in Ocean Island in October, 1948, will be described and the isolation of the virus reported elsewhere

A detailed study was made of one strain, isolated in the first passage in the form of amniotic fluid with an F/G titre of <10/480 when tested at room temperature When this fluid was titrated in eggs by amniotic and allantoic methods in parallel the results obtained were as shown in Table I

TABLE I —*Results of Passage of Ocean Island O Virus by Amniotic and Allantoic Routes at Different Dilutions*

Dilution of of inoculum	Amniotic route		Allantoic route	
	Number infected *	Phase †	Number infected *	Phase †
10^{-7}	1/5	0	—	—
10^{-6}	4/4	000	0/6	—
10^{-5}	8/8	000	0/6	—
10^{-4}	5/6	000	1/6	D
10^{-3}	—	—	4/6	δ
10^{-2}	8/8	000 D	5/5	δδδDD
10^{-1}	—	—	6/6	δδ DDD
10^{-0}	—	—	5/6	δδDD

* Number giving haemagglutination of guinea pig cells and number surviving
† Phase character of representative fluids (Burnet and Stone, 1945b)

It will be seen that the situation is similar to that reported for earlier strains in the O phase

Eighteen embryo lung emulsions which gave no agglutination of fowl cells at a dilution of 1 10 at room temperature were tested at 4° C Seven of these gave no agglutination at 1 10, with agglutinin titres for guinea-pig cells ranging from 100 to 320 Five others gave fowl cell titres at 4° C which were one-tenth or less of the guinea-pig cell titres These twelve can be regarded as true O phase virus Six lung emulsions gave the following values for fowl cell agglutination at 4° C, >80, >80, 80, 40, 40, 20 These last which gave no apparent agglutination at 100m temperature (18 to 20°) are referred to as " false O " phase virus As was pointed out in the paper by Burnet and Stone (1945b) it is impossible to draw sharp dividing lines in classifying the actual materials, amniotic fluid or embryo lung emulsion, from infected embryos Preparations can be obtained showing every graduation from " true O " which shows no fowl cell agglutination at any temperature to the full D phase

In vitro *Characteristics of " True O " Phase Virus in Contrast to " False O " Phase Virus*

Parallel experiments were carried out on (1) O and other variants of our standard strain BEL, which has been maintained in the O phase for seven years

(2) The strain IAN–O which might be called the most blatantly false O in our possession and (3) the new strain Ocean Island 1948 (Oc I)

Influence of temperature at which haemagglutinin titration is made

Our primary criterion of true O behaviour has been the absence of agglutination of fowl cells at 4° C by a 1 10 dilution of virus giving a titre of at least 80 with guinea-pig or human cells When false O strains, e g IAN–O, are studied at room temperature, close observation will show that in the early stages of settling a definite pattern is formed on the bottom of the tube By the time settling is complete there is at most only a slight roughness of the edge of the central " button " of cells If the cells are reshaken and again allowed to settle there is no trace of agglutination at any stage This finding, which is common to all the false O viruses tested, provides the clue to the understanding of the behaviour of these strains, viz that they very rapidly destroy by enzymic action the small proportion of fowl cell receptors to which they can be adsorbed Anything which prevents or slows down this elution of virus by its own enzymic action will increase the apparent haemagglutinating action on fowl cells

Apparent conversion of false O to D by treatment of the virus to reduce its enzymic activity

By appropriate treatment any influenza virus can be converted into an indicator strain, still producing haemagglutination to nearly full titre, but lacking enzymic activity and being inhibited by those mucoid materials which serve as a substrate for the untreated virus (Stone, 1949a) Simple heating will accomplish this with some strains, others require to be diluted in a mildly alkaline medium containing citrate or oxalate When these methods are applied to O and false O materials, the former show no development of a capacity to agglutinate fowl cells The false O viruses characteristically develop a power to agglutinate fowl cells at room temperature to a titre approaching that to which guinea-pig, human or pigeon cells are agglutinated The titre is never greater than that given by the untreated virus with fowl cells at 4° C Results with Oc I and IAN are shown in Table II

TABLE II —*Effect of Heat on the Agglutination Titres with Fowl and Guinea-pig Cells of True O and False O Virus*

			F/G titres at room temperature	
Strain	Phase	Treatment	Original	After heating
Oc I	O	56° C 45 min	<10/210	<10/80
Oc I	False O	,,	<10/430	40/160
IAN	,,	52° C 15 min	<10/120	140/120

With BEL false O strains, heating to 52° for 15 minutes in the presence of 1 per cent potassium oxalate allowed agglutination of fowl cells at room temperature, but the agglutination was of weaker type than that shown with guinea-pig cells Similar treatment of the true O phase virus produced no capacity to agglutinate fowl cells

The method used by Magill and Sugg (1948), suspension of virus in McIlwaine's phosphate-citric acid buffer at pH 5 6 was applied to the Oc I –O and false O

material used above (Table II) , F/G titres at room temperature produced by this treatment were <10/210 and 80/430 respectively

Very mild treatment of IAN false O, including incubation for an hour at 37° C in the presence of borate buffer pH 8 5 plus 0 5 per cent sodium citrate, allowed a full titre D type of agglutination of fowl cells at room temperature

Agglutination of fowl cells treated with periodate

Fazekas de St Groth (1949) has shown that at a certain level of periodate treatment red cells adsorb influenza viruses irreversibly, the normal enzymic action failing to occur   By treating a 25 per cent suspension of fowl cells with an equal volume of M/4000 KIO$_4$, cells were obtained which at room temperature were agglutinated by false O specimens of IAN or Oc I to the full titre, and by BEL false O to about half the titre obtained with normal fowl cells at 4° C   True O phase virus of either Oc I or BEL was without effect on such cells

The position in the receptor gradient of O and false O variants

Previous experience in testing O and D phases of the same strain for their position in the receptor gradient of cells, human or guinea-pig, susceptible to agglutination by both has generally shown that the two phases lie fairly close to one another, the O being earlier than the D (Burnet, McCrea and Stone, 1946) In view of the results described above it was of considerable interest to compare the position in the gradient for fowl cells of false O and D phases of the same strain   Since true O never shows any agglutination of fowl cells it cannot be tested   The experiment with BEL, false O and D, which is shown in Table III followed the usual plan of such tests   Fowl cells were treated with graded amounts of the vibrio filtrate, RDE, and the action stopped by the addition of citrate   After washing with saline the cells were made up to 1 per cent suspensions   Cells were also treated with BEL false O and NDV until stabilized   A modified series of viruses was then used to test the agglutinability of each type of cell at 4° C , five agglutinating doses (A D 's) being used   The false O material (BEL) was in the form of an embryo lung emulsion with a titre of 1600 with guinea-pig cells   The fowl cell titre at room temperature was <10, at 4° C , 200 It can be seen from Table III that BEL false O is at the " extreme left " of the gradient, quite separated from the D form of the same strain

TABLE III —*Relative Positions of BEL false O and BEL–D in Fowl Red Cell Receptor Gradient*

Red cells treated with	Haemagglutination by 5 A D 's at 4° C						
	BEL false O	NDV	MMF	WSE	BEL–D	SW	LEE
RDE							
1 10	—	—	—	—	—	—	—
1 50	—	—	—	—	—	—	+
1 250	—	—	—	—	++	++	++
1 1250	—	+	++	++	++	++	++
BEL false O	—	++	++	++	++	++	++
NDV	—	—	+	++	++	++	++
Control	++	++	++	++	++	++	++

DISCUSSION

The results reported in this paper will, we hope, clarify the confusion in regard to the genetic significance of the O–D series of changes in influenza A viruses   In a subsequent paper a detailed study of the enzymic activity of O, D and intermediate phases of BEL will be reported   This has given results in line with the interpretation which can be placed on the findings given in this paper

Our view is simply that when first isolated from human material influenza A virus particles are incapable of adsorption to and therefore of enzymic action on the receptor substance of fowl or mouse erythrocytes   In any large population of such virus particles a proportion of mutants appear which are capable of adsorption to the most " accessible " of the fowl cell receptor groupings   This proportion presumably is selectively favoured in the environment of the chick embryo and, in accordance with the now generally accepted views on microbial variation, further variants appear within the favoured clones by which the changed character is accentuated (Demerec, 1945)   As the change goes on the virus becomes capable of adsorption to progressively less accessible receptor groupings until it finally reaches its appropriate position in the fowl cell gradient of fully " egg-adapted" strains   In the early stages only a few receptor groupings are accessible and a brief period of contact between virus and cells results in their enzymic destruction with disappearance of the initial cell aggregation   Anything which hinders this enzymic destruction of the most accessible receptors will at this stage of virus adaptation produce an apparent conversion of virus from O to D   The simplest way to inhibit enzymic action is to lower the temperature, but the same result can be achieved by heating virus in the region of 52–56° C in an appropriate ionic environment, or by modifying the cell receptors with small amounts of periodate

Even if it were shown that " true O " virus as obtained in primary isolations by the amniotic method, could be induced to give fowl cell agglutination by some physical or chemical treatment, the data provided in this and earlier papers as to the conditions under which fowl cell agglutinating virus appears would still demand a genetic interpretation   The fact that any true O strain will, on passage, give intermediate and D descendants with complete regularity, yet cannot be induced by any manipulation *in vitro* to agglutinate fowl cells, wholly invalidates the view that the O–D phase variations represent merely transformations in agglutinating activity induced by the ionic environment

SUMMARY

Influenza A virus strains in true O phase as isolated from the human subject, cannot be manipulated by physicochemical means to produce fowl cell agglutination

The behaviour of intermediate forms which show such an apparent conversion is explicable in terms of their enzymic activity on cell receptors

The change from O to D phase on chick embryo passage is due to processes of mutation and selective survival

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AYFIVIN AN ANTIBIOTIC FROM *B LICHENIFORMIS* PRODUCTION IN POTATO-DEXTROSE MEDIUM

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MAGARÃO, Arriagada and Thales (1944) reported that cultures of *Myco tuberculosis* contaminated by a strain of *B subtilis* underwent lysis, and that products of the contaminating organism were able to modify the course of tuberculosis in guinea-pigs Cultures of the *B subtilis* which had been brought to Oxford were found to contain two species of aerobic spore-forming bacilli One was a strain of *B subtilis* and the other was a strain of *B licheniformis* When tested by the cross-streak procedure on nutrient agar both organisms inhibited the growth of *Staph aureus*, *C xerosis* and *Myco phlei*, but showed little activity against *Bact coli* or *Ps pyocyanea* Antibacterial substances produced by the strain of *B subtilis* have already been described (Newton, 1949) The present paper is concerned with an antibiotic formed in certain liquid media by the strain of *B licheniformis* This organism was originally called A-5 and the antibiotic has been named ayfivin (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949)

EXPERIMENTAL

The organism

The strain A-5 showed the usual characteristics of *B licheniformis* (Gibson, 1944) It was maintained for routine use on slopes of heart-extract agar, which were stored in the refrigerator until required Fresh subcultures were made every two weeks

Assay and unit of activity

Ayfivin was assayed by the cylinder-plate method (Heatley, 1944)   A laboratory strain of *Myco phlei* was at first used as a test organism, but this proved to be easily overgrown by contaminants and to be very sensitive to changes in pH   *C xerosis* was finally chosen as the most suitable test organism, and the assay was carried out on plates of Lemco-agar seeded with cultures of this organism which had grown for 24 hours at 37° C   The size of the zone of inhibition varied significantly with the pH of the agar, increasing as the pH was reduced from 8 to 6 5

The unit of ayfivin was based on the activity of a sample of crude ayfivin hydrochloride   This material was arbitrarily stated to contain 5 units per mg It was stored dry in the refrigerator and appeared to be quite stable   A solution of ayfivin containing 1 unit per ml produced zones of inhibition 18 to 21 mm in diameter on plates seeded with *C xerosis*   For the purposes of assay, solutions of ayfivin were diluted so that they contained from 0 25 to 1 unit per ml   and their activity was compared with that of the standard preparation

Medium and conditions of culture

The organism was grown in a potato-dextrose medium prepared in the following manner   King Edward potatoes were peeled, diced and steamed in tap water for one hour   The resulting mixture was filtered through lint, and glucose was added to the filtrate   Two hundred g of potatoes and 10 g of glucose were used for each litre of final solution   The initial pH of the medium was close to 7 0

The medium was distributed in glass culture vessels of the type described by Clayton, Hems, Robinson, Andrews and Hunwicke (1944)   Each vessel contained 500 ml of medium, which formed a layer 2 5 cm deep , the use of shallow layers of culture fluid appeared to be essential for good yields of the antibiotic

After autoclaving for 30 minutes at one atmosphere the medium in each vessel was seeded with about 2 ml of a 24-hour broth culture of A-5   It was found advantageous to use large inocula, and each vessel received 2 to 3 ml of a 24-hour shaken flask culture of the organism in heart extract broth ·

The seeded vessels were incubated at 37° C   The antibacterial activity of the culture fluid reached a maximum of from 4 to 8 units per ml after 5 to 6 days The final pH of the medium was about 7 2

FURTHER OBSERVATIONS

Crude ayfivin was extracted from the culture fluid obtained by growing A-5 in potato-dextrose broth (Sharp, Arriagada, Newton and Abraham, 1949)   The material was found to inhibit the growth of certain gram-positive bacteria in high dilutions and to have a relatively low acute toxicity to mice (Arriagada, Florey, Jennings and Wallmark, 1949)   It was therefore thought desirable to produce ayfivin in larger quantities and to make a fuller investigation of its chemical and biological properties   By arrangement with Dr D W Henderson large batches of active culture fluid were supplied by the Ministry of Supply Microbiological Research Department at Porton   Hills, Belton and Blatchley (1949) found that low yields of the antibiotic were obtained in medium prepared from some batches of potatoes, and they devised a synthetic medium which was more suitable than potato-dextrose broth for the production of ayfivin on a large scale

SUMMARY

A strain of *B lichenformis* isolated from a contaminated culture of *Myco tuberculosis* produces an antibiotic when grown in shallow layers of potato-dextrose broth   The antibiotic has been named ayfivin

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AYFIVIN PRODUCTION IN CHEMICALLY DEFINED MEDIA AND COMPARISON WITH LICHENIFORMIN

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AYFIVIN is an antibiotic produced by a particular strain (A-5) of *Bacillus licheniformis* (Arriagada, Savage, Abraham, Heatley and Sharp, 1949)   The present work deals with the development of a chemically defined medium suitable for the production of the antibiotic in bulk   Potato-dextrose medium had given good results when made from King Edward potatoes which had been stored for 6 to 10 months after harvesting (March to July, 1947), but, when this supply was exhausted, new potatoes of the varieties available at the time, or even King Edwards available later, gave only 25 per cent or less of the activity yielded by old King Edwards   The development of a reproducible medium, not subject to seasonal variation, was thus not only of importance for work on the biochemistry of antibiotic formation, but became an urgent practical necessity

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METHODS

In general, the techniques were those used by Belton, Hills and Powell (1949) in work on a chemically defined medium for the production of licheniformin by a different strain of *B licheniformis*, with modifications as follows

Maintenance of cultures

Spore suspensions were produced by growth on CCY agar (Gladstone and Fildes, 1940) for 4 days at 37° C   The growth was washed off in distilled water to give an opacity of 25 to 50 (Brown's standards)   After heating at 60° C for 90 min to kill vegetative forms the viable count was 2 to 5 × 10^9 spores per ml Such spore suspensions were prepared in quantities required for inoculation during 3 to 6 months and were stored in the refrigerator   Samples of the first suspension produced were also dried and stored *in vacuo* to preserve the strain in a form which had had as few subcultures as possible from the state in which it was originally received from Oxford

Culture media

Potato-dextrose —Batches of 45 l were made by scrubbing 20 lb King Edward potatoes, chipping and suspending in double muslin bags in 36 l tap water in a 15-gallon enamelled iron tank   After standing overnight the tank was steam heated at 90 to 100° C for 1 hour   The bag of potato residue was removed and 450 g glucose monohydrate was added to the liquor, together with tap water to restore the volume to 45 l   The pH was about 7 and was not adjusted further After standing for 30 minutes the fluid was filtered through one layer of lint or two layers of gauze, distributed in 2 l conical flasks (1 l per flask) and autoclaved at 7 lb /in ^{2} for 30 minutes

Chemically defined media —Ammonium salts and various sources of carbon, as described in the text, were added to a salt mixture containing $H_2PO_4^- - HPO_4^{--}$, 6 mM , citrate, 1 7 mM , $Mg^{++}, SO_4^{--}, 4$ mM , $Fe^{+++}, 70\mu M$, and $Mn^{++}, 13$ μM At first citrate was added as sodium salt (0 5 g /l) and phosphate as K_2HPO_4 (1 g /l), but later it was found desirable to modify this procedure (p 434)   The pH was adjusted to 7 2–7 4, but usually fell during autoclaving at 12 5–15 lb /in ^{2} for 20 minutes and was within the range 6 6–7 4 (glass electrode) at the time of inoculation

The chemicals used were not specially purified, but were of laboratory grade, available in quantity for moderate scale production

Conditions of cultivation

All cultures for production of antibiotic were incubated at 37° C in stagnant liquid media, the average depth being 10 mm   Pilot experiments were carried out with 15 ml quantities in 50 ml conical flasks   Production was with 1 l units in covered enamelled steel trays of dimensions about 25 × 40 cm

In flasks the most convenient method of inoculation was to add 10^5 spores/ml to the basal medium in bulk, followed by aseptic distribution   Separate flasks were used for each sample to be assayed, since it was found that disturbance of the pellicle by sampling led to irregular results on continued incubation   On the larger scale, the inoculum was pipetted into each tray in the hot room, followed by aseptic pouring of the culture medium, which had been sterilized separately

in resistance glass flasks   The trays were not subsequently moved before reaping   On this scale, sampling was possible without moving the tray and without appreciable disturbance of the pellicle   A proportionate inoculum of 10^8 spores per tray was unsatisfactory with the synthetic media, owing to the establishment of contaminating moulds during the long lag phase   The lag was reduced and contamination avoided by using an overnight vegetative culture (10 ml /tray) in tryptic beef digest broth (T M B)

Assay of antibiotic activity and its accuracy

Ayfivin —The cylinder-plate technique was used as developed by Arriagada *et al* (1949), except that tryptic beef digest agar (T M A) was used in place of Lemco agar   The test organism was the Oxford strain of *Corynebacterium xerosis*, and standard preparations of the active material were also supplied by the Oxford workers

All unknowns were assayed at two or three levels differing by twofold steps with at least three cups at each level   In single assays the standard was used at 0 5, 1 0 and 2 0 units/ml , and was treated similarly to the unknowns   In multiple assays an extra level of standard, 0 25 units/ml , was used and more cups were used at each level   Each cup contained 0 15 ml sample, and steps were taken to compensate for systematic errors arising from the tendency to smaller zones near the point of drainage of the surface inoculum (Heatley, 1944)   Bias was also recognized in multiple assays due to the cups not being filled simultaneously   Brownlee, Delves, Dorman, Green, Grenfell, Johnson and Smith (1948) compensated for this effect in streptomycin assay by an appropriate order of filling   In the present case a correction was determined   This was preferred, since, even for 12 assays set up together, it did not exceed $\pm$ 0 4 mm in the diameter of a zone, with a coefficient of variation of 20 per cent   The standard error of the deviation of each zone from the linear regression of zone diameter on log dilution was 0 5 to 0 9 mm (based on 25 to 125 degrees of freedom and not constant from day to day), so that the minimum significant difference between the zones for two assays, each the mean of six cups, was 0 6 to 1 1 mm   It is apparent that although the correction for the time of plating may be important, the error of its determination was an unimportant part of the total error and was usually negligible

Interpretation of data was usually carried out by graphical methods   In those cases where statistical examination was done, it was found that the slope of the regression line was not constant from day to day (6 5 to 8 5 mm change in diameter for ten-fold change in dilution)   Owing to association of greater deviations from regression with the steeper slopes, the accuracy of the determined potencies was relatively constant, the significant difference being 20 to 25 per cent of the larger value for assays based on six cups per sample carried out at the same time with plates of the same batch   For samples which were not compared directly, but only in terms of a standard preparation, due to determination at different times, the significant difference becomes about 33 per cent of the activity of the more potent material

Licheniformin —Assays were carried out by dilution in tryptic beef digest broth (T M B) as described by Callow, Glover, Hart and Hills (1947) with *Mycobacterium phlei* as test organism (Mill Hill strain)   The method is unsuitable for detection of differences in potency less than two-fold (Belton *et al* , 1949)

Chemical analysis of culture media

Sugar was determined by the method of Hagedorn and Jansen (1923), after hydrolysis, if necessary, with 0 25 N HCl 10 min at 100° C, lactate by the method of Friedemann and Graeser (1933) and ammonia using the still designed by Markham (1942)

<div align="center">RESULTS</div>

When work was begun on the A-5 strain of *B licheniformis*, licheniformin (Callow and Hart, 1946, Callow, Glover and Hart, 1947, Callow, Glover, Hart and Hills, 1947) was known to be produced by another strain (now N C T C No 7072) The synthetic medium for licheniformin production (Hart and Hills, 1947, Belton *et al*, 1949) gave less than 1 ayfivin unit/ml with the A-5 strain, while potato-dextrose then gave 2 to 4 units/ml The licheniformin-like activity given by the A-5 strain, however, was 700 dilution units/ml in the synthetic medium and only 90 units/ml in potato-dextrose The inverse relationship between the antibacterial activities of the two cultures of the same strain on different media, when compared by different methods, suggested that the principal activities are due to different antibiotics Since the N C T C No 7072 strain behaved similarly to the A-5 strain on both media, it is evident that the production of ayfivin or licheniformin-like materials is controlled largely by the nature of the medium rather than the strain Although both are identifiable as *B licheniformis* by the criteria of Gibson (1944), e g in failure to liquefy gelatin and capacity for anaerobic growth, the similar behaviour in antibiotic production is unlikely to be due to a common origin, they are easily distinguishable in colonial form, A-5 producing irregular mucoid colonies, 2 to 5 mm in diameter on T M A at 22 hours, while on the same medium N C T C 7072 produces smooth colonies at 16 hours, later giving rise to petal-like outgrowths which coalesce to form a dry radially striated periphery 4 to 7 mm in diameter (Fig 1)

Modification of Potato-dextrose

This medium usually had 22 5 to 26 5 mg /100 ml total N (16 to 19 mM) and 1 6 per cent total solids, including 1 per cent dextrose The synthetic medium, on the other hand, had about 60 mM NH_3 and 110 mM total lactate, corresponding to a solid content, including inorganic salts, of 1 65 per cent The low N content of the former medium accounted for the production of ayfivin, since addition of 100 mM ammonium chloride reduced the peak titre by 50 per cent (Table I), while replacement of the glucose by 100 mM ammonium lactate, to supply the same amounts of C and N as the glucose and ammonium chloride respectively, reduced the titre by 85 per cent Part of the effect in this case, however, may have been due to the high pH developed in the medium at an early stage, since replacement of the glucose by its C equivalent of sodium lactate led to a 70 per cent fall in the maximum titre with a rapid fall subsequently, and an even higher final pH Improvements in the technique of preparing the potato extract led to higher licheniformin titres than were observed in the preliminary experiments described previously, and these tended to be stabilized at a high level, rather than to fall when additional sources of N and C were supplied together In contrast, ayfivin production tended to show a more definite peak, falling rapidly if alkaline conditions developed with pH exceeding 8, and the highest peak values were observed with a high concentration of C source relative to the N supply

FIG 1 —*Bacillus licheniformis*   22 hours on tryptic beef digest agar   *a* Strain A 5   *b* strain
NCTC No 7072

Hills Belton and Blatchley

TABLE I —*The Effect of Additional Sources of C and N on Ayfivin Production in Potato Extract*

The concentration of all additions was 100 mM, except glucose, 50 mM

Days	Additions					
	None	NH₄Cl	Glucose	Glucose + NH₄Cl	NH₄ lactate	Na lactate
	Ayfivin, units/ml					
2	5	—	12	10	2	6
4	4	2	22	10	2	7
6	3	2	16	9	3	2
8	2	—	16	8	2	1
	Licheniformin, units/ml					
4	300	700	1500	800	1000	800
6	450	400	1000	1000	1250	450
8	450	—	500	1250	1250	450
	pH					
2	8 0	—	6 9	6 9	8 3	8 6
4	9 0	8 3	8 3	7 8	9 0	9 4
6	8 9	8 0	8 5	7 7	8 9	9 7
8	9 0	—	8 7	7 5	8 8	9 7

Modification of the Synthetic Medium

Proportion of N to C

Reduction of the proportion of ammonium lactate from 55 per cent to 20 per cent of the total lactate raised the peak ayfivin titre at 4 to 6 days from 2 to 5 units/ml , but the pH was then 9 2 and the titre subsequently fell considerably, as expected from the results with potato media   Replacement of the 100 mM sodium lactate in the modified medium by 50 mM glucose gave a peak titre of 14 units/ml at 4 to 8 days, falling to 9 and 5 units/ml at 10 and 12 days respectively, the pH falling from 8 7 to 8 4 during these latter stages of incubation

It thus appeared that it was possible to reach ayfivin titres in a synthetic medium approaching those previously reached in potato dextrose   Experiments on the optimum concentrations, however, soon showed that even higher titres were possible   The actual concentration appeared to be of less importance than the ratio of C/N, the optimum being in the region of 10 to 15   Various difficulties were encountered in attempting to determine the optima with precision   Thus when the constituents of the medium to be varied were autoclaved separately and mixed aseptically, growth was initiated with difficulty, especially at high glucose concentrations, so that with 120 mM glucose (2 4 per cent) and 108 mM ammonium lactate only 3 out of 5 flasks inoculated showed good growth in 4 days, while with 160 mM glucose and 144 mM ammonium lactate 1 out of 5 flasks showed growth in 4 days, and with 200 mM glucose and 180 mM ammonium lactate only

1 flask showed the first signs of growth at the 11th day and only 3 flasks had visible growth by the 19th day   On the same medium, however, all cultures which grew gave the same titre when tested after the same period from the initiation of visible growth, the optimum being 30 units/ml or over after 4 to 6 days visible growth with 120–200 mM glucose   This irregular growth was seen with media made up in either tap water or distilled water, and was reduced by adding starch equivalent to 40 mM glucose   It was eliminated completely by autoclaving the tap-water medium after mixing all constituents

Control of pH

This was important since little antibiotic was formed before the initial acid fermentation had ceased and the medium had returned nearly to neutrality , on the other hand, a high final pH was to be avoided because of the instability of the active material on incubation in alkaline media   These considerations had given no difficulty during production on potato-dextrose, since the pH at the time of reaping was usually 6 5 to 7 5   In the synthetic medium, however, the pH was usually 8 to 9 before the maximum titre was reached and it was considered that this might be the limiting factor in reaching high titres   Control of pH was

TABLE II —*The Effect of Buffer Concentration on Ayfivin and Licheniformin Production in Synthetic Media*

The C and N sources were approximately 120 mM glucose and 110 mM ammonium lactate

Days	mM K_2HPO_4		
	6	12	25
	Ayfivin, per cent geometric mean at 4 to 6 days with 6 mM K_2HPO_4		
2	7	3	0
3	78	55	36
4	107	92	87
5	103	74	82
6	90	66	67
	Licheniformin, units/ml		
4	1500	800	800
5	1500	600	800
6	1250	800	1000
	pH		
0	6 9	7 0	7 1
2	6 1	6 2	6 5
3	7 6	7 7	6 9
4	8 6	8 6	8 4
5	8 9	8 9	8 8
6	8 9	9 0	8 9

not easy in media containing such high concentrations of glucose (4 per cent) as were found to be optimal   Increase of buffer concentration to 4-fold merely delayed antibiotic production in certain media by retarding the return to neutrality after the initial development of acidity (Table II)   At 3 days the titres were significantly lower both with 12 mM buffer (P = 0 98) and with 25 mM (P > 0 999) Although $CaCO_3$ was better in reducing the initial development of acidity, and possibly allowing an earlier reaping, it was still necessary to determine the optimum reaping time with precision to avoid the rapid decline due to the subsequent development of alkalinity

Attempts were made to control the initial development of acidity by replacing the glucose partly or wholly by less rapidly fermented carbohydrates, starch, glucose and maltose, as with lactose in penicillin production   No significant effect was observed with starch   Table III shows that over the period 4 to 7 days, sucrose

TABLE III —*The Effect of Different Sugars on Ayfivin Production*

The ayfivin titres are expressed as per cent of the relatively constant value with sucrose at 4 to 7 days (geometric mean)   The values given are the geometric means for 2 experiments except at 7 days, where results from only one experiment were available   The observed differences between the values for the two experiments at 4, 5 and 6 days, excluding the small assays with lactose, showed no significant heterogeneity of variance (Bartlett's test , P = 0 4), and a pooled estimate, based on 21 degrees of freedom, was used in estimating the significance of differences

| Sugar | Concentration, mM | Ayfivin, as per cent that with sucrose | | | | | Probability of lower geometric mean due to chance |
		4 days	5 days	6 days	7 days	Geometric mean	
Glucose	100	46	86	85	60	68	0 01
Sucrose	50	87	97	119	100	100	—
Maltose	50	52	55	33	56	47	<0 001
Lactose	50	1	9	5	32	7	<0 001
Glucose	50	41	48	40	25	39	<0 001
Glucose 50 + sucrose	25	56	96	105	93	85	0 15
Glucose 50 + maltose	25	66	96	106	79	85	0 15
Glucose 50 + lactose	25	25	34	33	25	29	<0 001

was, on the whole, significantly better than its C equivalent of glucose (P = 0 99), mainly due to the earlier development of high activity , the apparent earlier decline with glucose was probably not significant (P = 0 07)   Lactose, as would be expected from fermentation tests in the *Bacillus* group, was not an effective source of C   Mixtures of glucose with either sucrose or maltose were not significantly different from either sucrose (P = 0 3) or glucose alone (P = 0 18)   Subsequently, when media were developed in which a high final pH was either avoided or delayed, sucrose showed no consistent advantage over glucose

This control of pH made use of the observation that cultures with ammonium chloride as sole source of N became very acid (pH < 5) and produced no anti-

biotic   If the amount of N supplied as ammonium lactate was insufficient and the remainder was supplied as ammonium chloride, a suitable balance of these two salts ensured a final pH which did not rise above 7 (Table IV)   No improvement in the peak antibiotic titre occurred, however, until the sodium citrate and potassium phosphate of the basal medium were replaced by the acids or their ammonium salts, the adjustment of the initial pH of the medium being made with ammonia rather than NaOH   Na$^+$ and K$^+$ were supplied as chlorides

TABLE IV —*The Effect of Ammonium Chloride on Antibiotic Production and pH Changes*

All media had 200 mM glucose

		Medium A 1 7 mM Na$_3$ citrate 5 0 mM K$_2$HPO$_4$			Medium B 1 7 mM Am$_3$ citrate 5 0 mM NaCl 5 0 mM Am$_2$ HPO$_4$ 10 0 mM KCl		
Ammonium	Chloride	33	33	0	33	33	0
salts, mM	Lactate	40	80	80	40	80	80
Days		Ayfivin, units/ml					
3		8	5	8	5	13	12
4		8	16	26	9	20	22
5		33	34	34	32	47	47
6		23	21	24	28	33	38
		pH					
0		6 7	6 1	6 3	6 7	6 7	6 3
3		5 3	6 0	6 0	5 6	5 4	6 4
4		5 5	6 4	7 0	5 7	6 0	7 4
5		4 9	7 4	7 9	5 9	6 1	7 9
6		5 9	8 2	8 5	6 2	6 3	8 3

Optimum concentrations of glucose and ammonium lactate

These optima were interdependent as shown in Fig 2 to 4, which incorporate the results of 7 independent experiments   Fig 2 shows the concentrations, in the presence of ammonium chloride, at which the maximum titres equalled or exceeded 10, 20 and 30 Oxford units/ml respectively   In order to avoid giving excessive weight to exceptionally high assays, the maximum has been taken as the geometric mean of the two consecutive highest daily titres in the period 4 to 6 days, or the geometric mean of all three where these did not differ significantly   At low concentrations of glucose and ammonium lactate, the maximum was reached in 4 to 5 days, while at high concentrations 5 to 6 days were usually necessary   Fig 3 and 4 show some of the actual values observed, as opposed to the limits within which they occur, at selected concentrations   With glucose concentrations in the neighbourhood of 125, 200 and 265 mM, the optimum ammonium lactate concentrations were 35, 50 and 80 mM respectively, but the upper limit was not
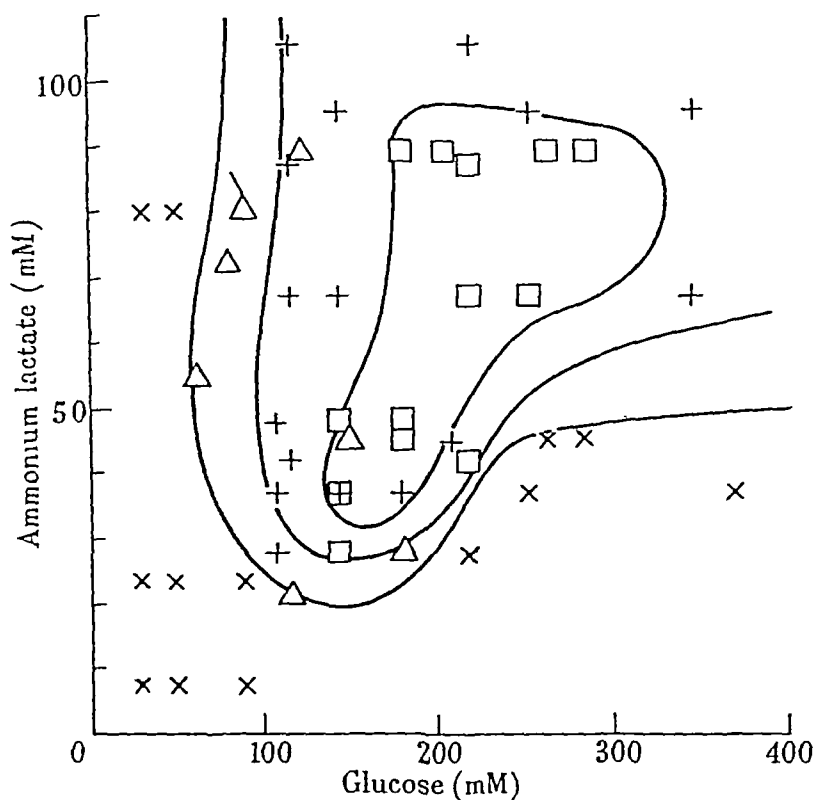
FIG 2 —The effect of glucose and ammonium lactate concentrations on ayfivin production in the presence of ammonium chloride   Less than 10 units/ml × ,   10–20 units/ml △ (single obs) , ⋏ (two identical observations) ,   20–30 units/ml + ,   over 30 units/ml □ The curves show the concentrations at which the maximum yields were 10, 20 and 30 units/ml
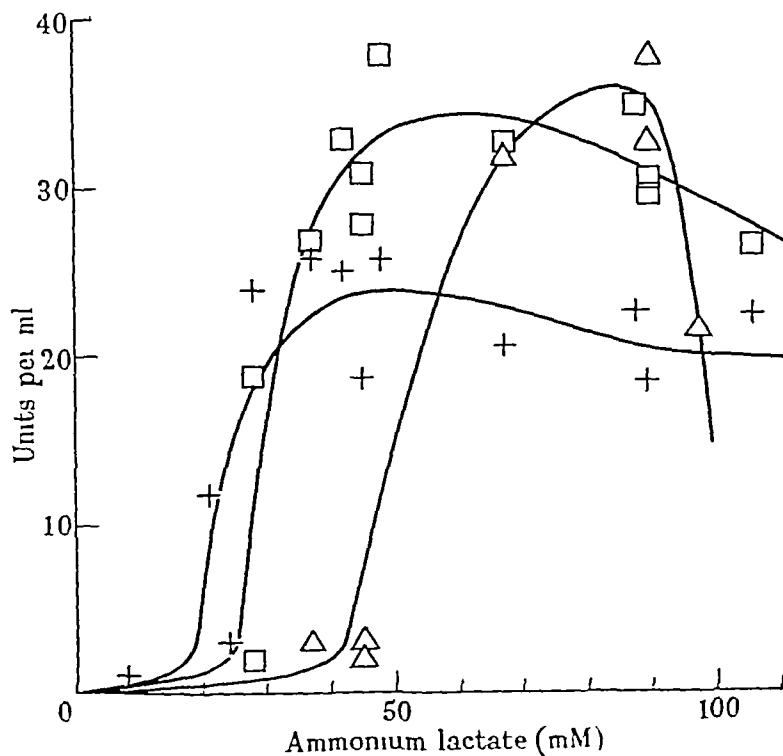
FIG 3—Optimum ammonium lactate concentrations for ayfivin production at different glucose concentrations in the presence of ammonium chloride   Glucose concentration (mM) 125 +   200 □ , 265 △

sharply defined, except possibly at the highest glucose concentration (Fig 3)
The optimum glucose concentration was more definite (Fig 4), and increased only
from 190 mM to 260 mM as the ammonium lactate concentration increased from
40 to 80 mM, but the titres were most sharply limited by excess glucose at low
lactate concentrations   These critical concentrations extended from the region
of 30 mM lactate and 180 mM glucose to 45 mM lactate and 250 mM glucose, so
that combinations of lactate and glucose concentrations, lower and higher respec-
tively than this boundary, gave practically no antibiotic due to acid formation
(pH 4 5 or less) which was not reversed on further incubation
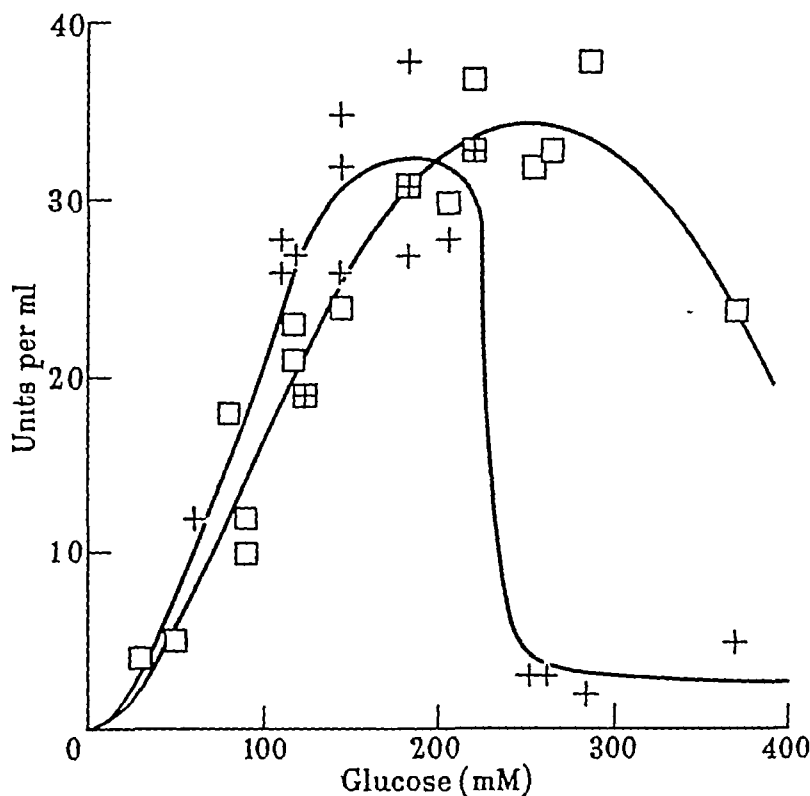
Fig 4—Optimum glucose concentration for ayfivin production at different ammonium lactate
concentrations in the presence of ammonium chloride   Ammonium lactate concentration (mM)   40
+ , 80 □

Consumption of Nutrients

Table V shows the consumption of glucose and ammonia in parallel with ayfivin
formation and pH changes at ammonium lactate concentrations 28 to 89 mM with
approximately 180 mM glucose (Exp 1) and with 45 mM ammonium lactate and
163 to 285 mM glucose (Exp 2)   Exp 1 also shows the changes in lactate con-
centrations in some cases   Although much of the glucose was consumed before
high ayfivin titres were reached, at least 10 μmol/ml usually remained at the
time of maximum antibiotic production   Consumption was delayed at glucose
levels above 180 mM and considerably so at 285 mM   Ammonia consumption,
on the other hand, was less influenced by its initial concentration or that of glucose,
except at high glucose levels (285 mM) in the presence of insufficient ammonium

TABLE V —*The Consumption of Nutrients during Ayfivin Production*

All media contained approximately 30 mM ammonium chloride

Experiment	Initial concentration (mM)		Days	Ayfivin (units/ml)	Change in nutrients (μ mol /ml)			pH
	Glucose	Lactate			Glucose	Lactate	NH₃	
1	180	28	0	—	—	—	—	7 5
			3	3	−101	+17	−34	5 1
			5	4	−162	+19	−34	4 6
	180	36	0	—	—	—	—	7 2
			3	15	−143	+14	−45	5 5
			5	21	−171	−12	−66	5 1
	180	49	0	—	—	—	—	7 4
			3	23	−148	−1	−52	5·8
			5	40	−172	−30	−69	5 3
	180	89	0	—	—	—	—	6 7
			3	8	−113	—	−46	6 5
			5	22	−171	—	−82	8 2
2	163	45	0	—	—	—	—	6 9
			3	4	−104	—	−39	6 0
			5	31	−151	—	−60	5 0
	205	45	0	—	—	—	—	6 8
			3	5	−90	—	−35	6 1
			5	27	−186	—	−59	5 9
	285	45	0	—	—	—	—	6 7
			3	3	−36	—	−23	6 2
			5	3	−156	—	−40	4 7

lactate (45 mM) to allow antibiotic formation Good antibiotic titres were reached with a consumption of glucose of 150 to 190 μmol/ml and of ammonia 60 to 80 μmol/ml At these levels lactate consumption was 10 to 30 μmol/ml , but was replaced by accumulation of lactate where the utilizable ammonia was inadequate in relation to the available supply of glucose It may be concluded that about 15 atoms of C were required for the utilization of 1 atom of N under conditions where good ayfivin production occurred

Application to Production

Before conditions were found for preventing the final alkaline drift of the culture, production of 200 l batches was begun on a synthetic medium containing 32 g glucose monohydrate and 22 5 ml 50 per cent ammonium lactate per l Analysis of the autoclaved medium gave about 155 mM glucose, 125 mM lactate and 145 mM ammonia The assay of 10 consecutive batches, ranging from 8 0 to 13 4 units/ml , had a geometric mean of 10 26 Of these batches, 5 reaped at 4 days gave a geometric mean of 10 33 not significantly different (P ≈ 0 9) from the 10 19 for 5 reaped at 5 days These values were also not significantly different from that observed with potato-dextrose medium, 10 1, in 28 consecutive batches, ranging from 6 5 to 14 7 units/ml The glucose concentration in the synthetic medium was believed to be suboptimal, but higher concentrations were avoided

because they delayed growth, allowing contaminating moulds to become established —a problem which was serious with a light spore seeding, but which was overcome by the use of a heavy vegetative inoculum In smaller aliquots (15 ml /50 ml conical flask) there was a rise in titre after the 4th day to about 23 units/ml at 5 and 6 days, but in larger cultures with the same average depth of medium the values were falling at 6 days The reasons for this discrepancy have not been investigated

Following the use of ammonium chloride to control the final pH of the medium, higher titres were reached on the larger scale approaching those reached in small flasks The optimal concentrations of ammonium lactate and glucose were found to be similar to those previously observed on a smaller scale and K^+ was found to be essential, but not Na^+ The optimal concentration of KCl was reached at 7 mM (Table VI) , in Exp 1 lower yields of antibiotic were observed at 2 mM, both with and without Na^+, but Exp 2 showed no significant heterogeneity with 5 concentrations from 2 to 16 mM (P = 0 35 for 4th day)

TABLE VI —*Optimum Potassium for Ayfivin Production*

			Ayfivin, units/ml with—				
Experiment	NaCl (mM)	Days	2	4	7	10	16 mM KCl
1	0	4	12	—	21	—	21
		5	12	—	21	—	17
		6–7	7	—	22	—	17
	11	4	14	—	26	—	—
		5	18	—	29	—	—
		6–7	10	—	26	—	—
2	7	4	12	16	17	12	16
		5	18	15	15	16	16
		6–7	21	17	20	17	21

The medium finally adopted therefore had the following composition

Glucose monohydrate	44 g
Ammonium lactate (5 25 M)	10 ml
$MgSO_4$ $7H_2O$	1 g
Iron ammonium citrate (1 7 per cent)	1 ml
$MnSO_4$ $4H_2O$ (0 3 per cent)	1 ml
Ammonium chloride	0 6 g
Sodium chloride	0 4 g
Potassium chloride	0 4 g
Phosphoric acid (sp gr 1 75)	0 3 ml
Citric acid	0 3 g

Dissolved in tap water and made up to 1 l

Ammonia (sp gr 0 880)   approx 1 2 ml to bring pH to 7 3 to 7 4

After autoclaving (12 5 to 15 lb /in ^{2} for 20 minutes), the analytical figures were 195–205 mM glucose, 54–57 mM lactate and 75–90 mM ammonia, pH 6 3–7 3 The figures for lactate were reproducible only if the strength of the commercial 50 per cent W/W solution was standardized before use Analytical control of

the other components was not found to be essential since the concentrations were not critical

After reaping at 5 days, 5 consecutive batches, ranging from 12 to 32 units/ml, gave a geometric mean of 20 8 significantly greater (P = 0 99) than either the earlier synthetic medium or potato dextrose At 4 days, although high titres were sometimes reached, the geometric mean, 12 8 units/ml, was not significantly higher than that with earlier media, owing to the scatter, 6 6 to 35 units/ml, of the observations Any improvement which occurs on further incubation is relatively small, so that not less than 10 to 20 batches would be necessary to establish it with a high degree of probability

Differentiation of Ayfivin from Licheniformin

Although licheniformin titres of 1500 units/ml, as high as any previously observed, were reached in some media designed for ayfivin production (Table II), the ratio of licheniformin units to ayfivin units never exceeded 50, compared with about 1000 for a purified sample of licheniformin supplied by Dr R K Callow In the medium finally adopted, a ratio 3 was more usual and even lower values were observed Although the cultures were thus practically free from licheniformin (and even the small activity which was observed against the licheniformin test organism may not have been due to the same active principle), it was desirable to establish the differentiation on a firmer basis

Table VII shows the relative effects of Seitz-filtration and autoclaving, both at pH 2 5 on the activities of culture fluids for licheniformin and ayfivin production, and of purified preparations The samples were assayed by the cylinder-plate technique against *C xerosis* Licheniformin showed a variable loss on

TABLE VII —*The Effect of Seitz-filtration and Autoclaving on Activity of Ayfivin and Licheniformin-like Preparations*

Filtration and autoclaving were carried out at pH 2 5 and the samples were readjusted to pH 7 before testing In filtration, 10 ml were passed through a Ford Sterimat SB 3 6 cm in diam Autoclaving was at 10 lb / in² for 12 5 min

Assays were carried out by the cylinder plate technique with *C xerosis* as test organism

		Per cent recovery	
Antibiotic	Type of preparation	Filtered	Autoclaved.
Ayfivin	{ Culture fluid	86	49
	{ Partially purified*	87	58
Licheniformin	(Culture fluid, A-5 strain, 875 units/ml	59	229
	} Fluid from autoclaved culture, A-5 strain, 2000 units/ml	91	—
	(Partially purified	95†	104†
		—	90‡

* From Dr E P Abraham, School of Pathology, Oxford, and used at 0 5 mg /ml = 11 Oxford units/ml

† From Dr R K Callow, National Institute for Medical Research using strain N C T C 7072 The concentration was 1·0 mg /ml = 5000 dilution units/ml

‡ From Mr L H Kent and Dr B T Tozer of this department using strain A 5 The concentration was 0 2 mg /ml = 1000 dilution units/ml

filtration   The autoclaved culture fluid and purified preparations, which had been isolated from fluid which had been boiled, both showed no significant loss Unheated culture fluid, however, showed about 40 per cent loss   At present it is not known whether this discrepancy is due to a change in the nature of the material, or whether it is merely due to the increased amount which appears to be produced in the culture fluid as a result of heating   Apart from this increase, changes on autoclaving were insignificant   With ayfivin, on the other hand, there was about 15 per cent loss on filtration under the conditions used and about 50 per cent loss on autoclaving

The two antibiotics also differ in their diffusibility in agar and in their antibacterial activity against various test organisms   Thus, although good culture fluids for the preparation of either antibiotic inhibit C xerosis in broth at a dilution of about 1/2000, fluids for ayfivin production must be diluted 20- to 40-fold to give the same size of zone as 1 unit/ml Oxford standard in the cylinder-plate assay, while licheniformin culture fluids give the same size of zone without dilution   These additional dilution factors should be borne in mind in considering the inhibitory dilutions against various test organisms of preparations standardized by the cylinder-plate method (Table VIII)   The correspondence between those values given for C xerosis in broth and on the surface of agar shows that the differences between the values for ayfivin and licheniformin for this organism are due to different rates of diffusion, and not to a different type of action against organisms in broth as compared with those grown on the surface of agar   There is good agreement, within the limitations of the methods, between the values

TABLE VIII —*Antibacterial Activity against* C xerosis *and strains of* Myco phlei *of Ayfivin and Licheniformin-like Materials Standardized by Diffusion Method*

All materials were sterilized at pH 2 5 either by Seitz-filtration or by autoclaving 12 min at 10 lb /in ² and were adjusted aseptically to pH 6 5–7 5 before testing   The preparations were the same as those of Table VII

The diffusion unit is the amount of material per ml of a solution which gives the same size of zone, in the cylinder plate assay with C xerosis, as 1 unit/ml of an Oxford standard of ayfivin

The dilution unit is the amount which in 1 ml culture medium just inhibits a chosen test organism   Culture media were tryptic beef digest agar (T M A) and tryptic beef digest broth (T M B)

The range of the ratio dilution units/diffusion units is given for at least four independent observations

	Ratio, dilution units/diffusion units				
	Corynebacterium xerosis		Mycobacterium phlei		
Antibiotic	T.M.A	T.M.B	N C T C No 1471 T M B	Mill Hill T M B	N C T C No 525 T M B
Ayfivin					
Culture fluid	—	90–120	30–110	4–40	2–15
Partially purified	40–45	40–160	20–80	1–15	0–1
Licheniformin					
Culture fluid, A-5 strain	—	400–1200	800–2000	200–1400	800–1600
Partially purified, A-5 strain	600–2000	800–2000	2500–4000	450–900	1600–2400
Partially purified, N C T C No 7072	900–1100	600–3000	500–5000	350–1200	1000–7500

for culture fluids and the corresponding purified materials, showing little qualitative change as a result of isolation   The licheniformin from strain A-5 is similar to that from N C T C 7072, both inhibiting the strains of *Myco phlei* used, at dilutions little different from those inhibiting *C xerosis*   With ayfivin, on the other hand, the Mill Hill strain is relatively resistant and N C T C 525 is quite resistant, except to culture fluids which may contain traces of licheniformin

DISCUSSION

The main finding of this work is that the antibiotic activity of culture fluids of *Bacillus licheniformis* is altered qualitatively as well as quantitatively by the nature of the culture medium   Although other species of organism are known to produce more than one antibiotic, little is known of the part played by the culture medium except in the case of the penicillin family, where the presence of derivatives of phenyl acetic acid is known to favour, under suitable conditions, the production of penicillins with an aromatic sidechain (Higuchi, Jarvis, Peterson and Johnson, 1946)   Preparations from different media are also stated to show differences with both tyrothricin (Stokes and Woodward, 1943) and subtilin (Lewis, Humphreys, Thompson, Dimick, Benedict, Langlykke and Lightbody, 1947), but the constituents responsible have not been defined   With *Bacillus licheniformis* it has now been shown that definite changes in synthetic media give rise to culture fluids and concentrates with different antibiotic properties, but it is not yet possible to account for these differences in terms of the chemical structures of the antibiotics concerned   The effect of the medium, however, is not likely to be related to the presence of precursors of specific groups, as with the penicillin family, since the changes may be produced simply by the use of glucose instead of lactate as main source of C with a reduction in the ratio of utilizable N/C from 1/6 to 1/15, the N being supplied as ammonia in both cases   On the medium with lactate and a high N/C ratio, which had previously been designed for the production of licheniformin by strain N C T C No 7072 (Hart and Hills, 1947 , Belton, Hills and Powell, 1949), licheniformin-like activity was also produced by the A-5 strain, and an active hydrochloride was isolated (Kent and Tozer, unpublished) by the method of Callow *et al* (1947)

On the medium with glucose and a low N/C ratio, the active principle produced by both strains was less stable to autoclaving at pH 2 5 and less active against two more or less resistant strains of *Myco phlei* (N C T C 525 and Mill Hill), but was relatively more active when assayed by a diffusion method against *C xerosis*, presumably owing to greater diffusibility in agar   Concentrates of material from the A-5 strain were characterized as ayfivin (Sharp, Arriagada, Newton and Abraham, 1949)

It is possible that the effect of the C source was related to the pH changes associated with its metabolism rather than the direct effect of a particular chemical structure   Control of pH was certainly important in ayfivin production, but owing to the high concentration of glucose required, the use of buffers or chalk was not entirely satisfactory and periodic aseptic adjustment was impracticable with stagnant, pellicle-forming cultures   The principle finally adopted was the use of balanced concentrations of acidic and basic metabolites   This has already been applied, in a more elaborate form, to the development and maintenance successively of optimum pH values, first for growth of *Penicillium chrysogenum*, and subsequently for penicillin production by the mycelium (Jarvis and Johnson,

1947) Since success in the use of ammonia as source of N depended on choice of concentration and control of pH, reported failures in the cases of other antibiotics may have been due to such causes This is certainly the case with subtilin, which has now been produced with ammonia as sole source of N (Feeney, Garibaldi and Humphreys, 1948), in spite of earlier reports to the contrary (Jansen and Hirschmann, 1944) With tyrothricin (Lewis, Dimick and Feustel 1945) although the medium was improved by supplementing N of ammonium sulphate with a smaller proportion from tryptone, good titres were only reached on this type of medium when citrate or malate were also added, although citrate was strongly inhibitory with tryptone as sole source of N The metabolism of these anions would counteract the acidity arising from utilization of ammonia

In view of the importance of comparatively small modifications, such as changes in concentration or rigid control of pH, in changing not only the amount but even the nature of the antibiotic produced by *Bacillus licheniformis*, much of the investigation of culture collections for antibiotic organisms may be of limited value due to the use of unsuitable media Experience with *B licheniformis* suggests that ordinary laboratory media designed for optimal growth tend either to produce little antibiotic or to do so transiently, the optimum time for harvest being limited and variable Better results have been attained in media in which growth is initiated with difficulty, as with the A-5 strain at high glucose concentrations, and in which the nutrients are minimal for satisfactory growth Consequently we are inclined to favour the view that antibiotics may be either by-products of metabolism or products of incomplete metabolism which do not accumulate under conditions most favourable for the producing organism An analogy is furnished by diphtheria toxin production which is optimal at Fe concentrations suboptimal for growth (Pappenheimer, 1947) The detailed mechanism by which the medium influences the nature of antibiotic formation has not so far been studied Apart from a direct chemical effect on metabolism, the possibility arises of causing mutation or of favouring the selection of particular variants which may pre-exist in the inoculum Investigation of this problem is being continued by other workers in this department

SUMMARY

A synthetic medium has been devised to replace potato-dextrose for production of the antibiotic, ayfivin, by a particular strain of *Bacillus licheniformis* The best synthetic medium produced 12 to 32 units/ml in 5 days at 37° C , compared with 2 to 15 units/ml in 4 to 6 days on the potato-dextrose medium, subject to variation due to season and variety

The conditions for ayfivin production were a high C/N ratio of 15 and maintenance of pH in the region of neutrality These conditions were satisfied in stagnant culture in a medium containing inorganic salts 0 2 M glucose, 0 05 M lactate and 0 08 M ammonia

The inorganic requirements were not studied in detail but K^- was shown to be essential at a concentration of not less than 2 mM (80 p p m) and Fe^{+--}, Mg^{--} and Mn^{+-} were always added

On a similar medium with a lower C/N ratio of 6, and C supplied as organic anions which were metabolized to give a high pH a different antibiotic was produced with licheniformin-like characteristics shown by lower diffusibility in agar, greater stability and greater potency against some strains of *Mycobacterium phlei*

We wish to thank Sir Howard Florey for introducing this problem to us, and Dr N G Heatley for help in standardizing the method of assay Dr E P Abraham and Dr D W Henderson have contributed valued criticism Permission to publish has been granted by the Chief Scientist, Ministry of Supply

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AYFIVIN EXTRACTION, PURIFICATION, AND CHEMICAL PROPERTIES

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THE production of ayfivin by the strain A–5 of *B lichemformis* has been described by Arriagada, Savage, Abraham, Heatley and Sharp (1949), who grew the organism in potato-dextrose broth, and by Hills, Belton and Blatchley (1949), who later devised a more convenient synthetic medium   A method by which preparations of ayfivin with high antibacterial activity have been obtained from the culture fluids of the *B lichemformis* is given in this paper   The chemical properties of the preparations indicate that ayfivin is a polypeptide

EXPERIMENTAL

Extraction

Preliminary experiments showed that ayfivin was not extracted from the culture fluid by common organic solvents other than butanol or phenol Adsorption on to charcoal was therefore used as the first step in its isolation Elution was accomplished by the use of a two-phase mixture of butanol and dilute hydrochloric acid   The ayfivin in the eluate was transferred from water to butanol, and back to water, by appropriate adjustment of the pH of the solutions, and was then precipitated as a picrate

In the course of these experiments the potato-dextrose medium was replaced by the synthetic medium of Hills *et al* (1949)   No significant difference was noticed in the behaviour of the active material from the two types of culture fluid during the course of purification

Adsorption on to charcoal

The culture fluid was clarified in a Sharples centrifuge and the resulting fluid was stirred for 10 minutes with about 0 75 per cent (w/v) of active charcoal (Farnell Grade 14 neutral or Grade LS neutral)   The charcoal was then separated in a Sharples centrifuge or on a large filter   Different charcoals varied greatly in their suitability, with certain acid charcoals no active material could subsequently be eluted   The exact amount of charcoal used depended on the culture fluid   The best results were obtained when the amount was sufficient to adsorb from 90 to 95 per cent of the activity   If larger quantities were used, elution became more difficult and the final yield was reduced, presumably because a larger proportion of the antibiotic was then held on the most strongly adsorbing centres

Elution

Ayfivin was eluted by stirring the charcoal corresponding to each 100 l of culture fluid with a mixture of 1 l of *n*-butanol and 4 l of 0 2 N aqueous hydrochloric acid. Butanol was adsorbed preferentially by the charcoal from the two-phase mixture. After filtration the eluate consisted almost entirely of water saturated with butanol. The yield of ayfivin in the eluate was from 40 to 50 per cent of that present in the culture fluid.

The partition coefficient [ayfivin in 0 2 N HCl]/[ayfivin in *n*-butanol] is about 10. In consequence, ayfivin eluted by the butanol is largely displaced into the aqueous phase. Here it is excluded from direct contact with the charcoal since the latter is covered by a layer of butanol. The use of the two liquid phases thus greatly favours elution.

A method of eluting substances from charcoal with a mixture of water and an organic solvent immiscible with water, such as benzene, was described by Steenberg (1944). The procedure differed from that used with ayfivin in that the substance to be desorbed was insoluble in the organic solvent by which it was displaced from the charcoal. Ayfivin, which is insoluble in ether and in benzene, was only eluted from charcoal in very poor yield by a mixture of water and benzene, or water and ether.

Little ayfivin was eluted by a solution of 0 2 N HCl saturated with butanol, or by butanol saturated with aqueous 0 2 N HCl. Acid ethanol was at one time used, but loss of activity occurred during concentration of the relatively large volume of solvent required for elution.

Transfer between water and butanol

The eluate was adjusted to pH 7, saturated with sodium chloride, and shaken with one-fifth of its volume of butanol, which extracted the ayfivin. One fifth of its volume of water and two volumes of ether were mixed with the extract, and the pH of the aqueous phase was adjusted to 2 with hydrochloric acid. The aqueous phase, which then contained the ayfivin, was freed from dissolved butanol by extraction with ether, and its pH adjusted to 5.

Precipitation of crude ayfivin picrate

The aqueous solution was cooled in ice, and a saturated aqueous solution of picric acid was added until there was no further precipitate of ayfivin picrate. The picrate was filtered and dried *in vacuo*.

Crude ayfivin hydrochloride

The picrate was decomposed by dissolving it in 0 2 N ethanolic hydrochloric acid. Ayfivin hydrochloride formed a white flocculent precipitate on adding dry ether to the solution. It contained 20 to 30 units/mg. The unit of ayfivin is described by Arriagada, Savage, Abraham, Heatley and Sharp (1949).

Further Purification   Counter-current Distribution Between Solvents

A number of methods were investigated for the further purification of crude ayfivin. They included precipitation from aqueous solution by salts, chromatography under various conditions, ionophoresis in silica gel (Consden, Gordon and Martin, 1946), and counter-current distribution between solvents. The method

of counter-current distribution between solvents, developed in recent years by Craig and his colleagues (Craig, 1944), was the most promising   Some preliminary results with this method are described here

Ayfivin appeared to have an isoelectric point at about pH 7 1, and measurements of antibacterial activity showed that its partition coefficient between water and butanol varied rapidly in this region (see below)   It was therefore thought possible that the components of crude ayfivin might be separated most efficiently in a system with a pH near to 7   Subsequent experiments showed a striking difference between the amount of resolution obtained in neutral and in acid solution

*Distribution between an amyl alcohol-*n-*butanol mixture and water or phosphate buffer at pH 7*

The amounts of activity found in the two phases when crude ayfivin (from 0 5 mg /ml to 10 mg /ml) was distributed between water or $\frac{m}{10}$ phosphate buffer, at pH 7, and a mixture of amyl alcohol and n-butanol (4 1) indicated that the overall partition coefficient of the active material was close to unity   The activity of crude ayfivin could be raised by eight-tube counter-current distributions in this system   Different batches of ayfivin contained varying amounts of material of low specific activity which congregated at both ends of the series In general, the most active fractions (of about 30 to 40 units/mg) were found in Tubes 3, 4, and 5   The accuracy of the method of assay was too low to make an exact analysis of the results on the basis of activity   Nevertheless, comparison of the experimental distribution curve for the activity with theoretical curves for a single substance indicated that the material contained significant amounts of three active substances   A major active constituent with a partition coefficient close to 1 showed a maximum near the centre of the series, while minor constituents appeared nearer the ends of the series   Different batches appeared to contain somewhat different amounts of the minor active constituents   The results of experiments with two batches are described below

(1) Using separating funnels to hold the solvents, several grammes of crude ayfivin could be purified in one experiment   For preparative purposes the material in each funnel was transferred to the water phase at the end of the distribution, by adjusting the pH to 2 with hydrochloric acid and adding three volumes of ether   The pH of the aqueous solutions was then brought to 5 with sodium hydroxide, and the material from the different funnels precipitated in the form of the relatively insoluble picrate

Fig 1 illustrates the distribution of activity and of total material when one batch of ayfivin (Batch A) was subjected to eight transfers in a system composed of equal volumes of mutually saturated amyl alcohol—n-butanol (4 1) and water The distribution was begun with 2 g of ayfivin (22 units/mg) and the aqueous phase (50 ml) adjusted to pH 7 , the alcohol phase was mobile   No change in pH was detected during the distribution The material appeared to contain three active constituents, which contributed about 15 per cent, 80 per cent and 5 per cent, to the total activity and concentrated in the tubes near the beginning, the centre and the end of the series respectively   The specific activity of the major constituent in Tubes 3, 4, and 5 was about 40 units/mg   Calculations of partition

coefficients from the relative amounts of total material in adjacent funnels (Williamson and Craig, 1947) did not indicate that any of the material was homogeneous

(2) The results of an eight-tube distribution on a smaller scale and with a different sample of ayfivin (Batch B) are shown in Fig 2   The crude material, which had an activity of 20 units/mg , was part of a large batch that has been reserved for more extensive distributions
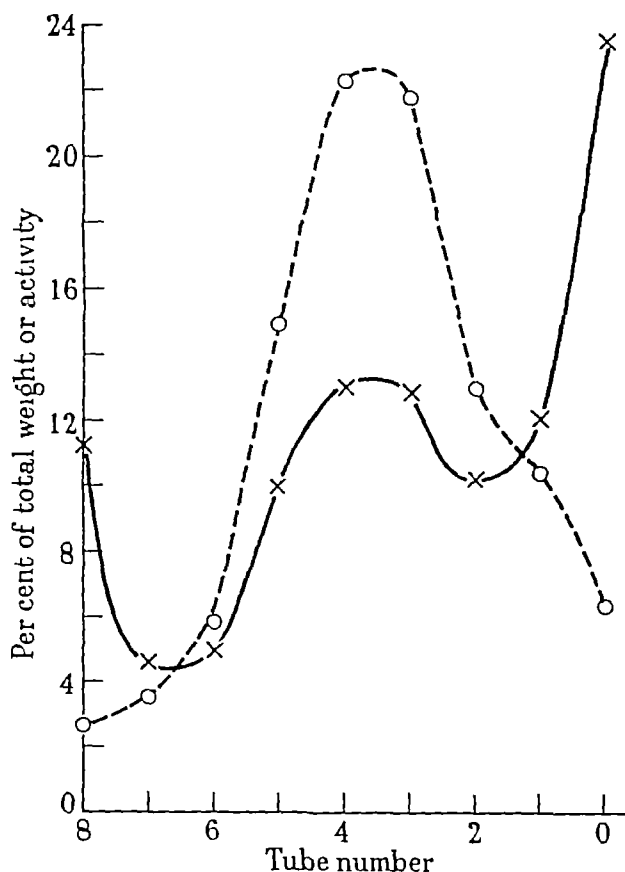
FIG 1 —Eight tube counter current distribution of crude ayfivin (Batch A) between amyl alcohol *n* butanol (4 1) and water (pH 7)   Upper phase mobile

-×————————×- Dry weight
-○--------○ Activity

The experiment was carried out in stoppered test-tubes   The two phases, each of 8-ml , were amyl alcohol-*n*-butanol (4 1) and M/10 phosphate buffer at pH 6 8   The crude ayfivin hydrochloride (100 mg) was dissolved in a mixture containing rather more than 8 ml of each phase, and the pH was adjusted to 7 with N NaOH   The volume of each phase was made up to 9 ml , and a small amount of insoluble material was removed by centrifuging   Tube 0 was then filled with 8 ml of each clear layer, and the remaining 2 ml of the mixture was used for control determinations of dry weight and activity   The phosphate phase was mobile   At the end of the distribution 0 1 ml amounts of both phases were removed from each tube and added to water (2 to 4 ml) for determinations of

activity   The alcohols were extracted from the solutions by *n*-hexane before assay   The remaining alcohol phases were then removed, and the aqueous layers were extracted first with 3/5 volumes and then with 2/5 volumes of *n*-butanol Each extract was mixed with the original alcohol phase from the corresponding tube   The weight of material in the extracts from each tube was determined by evaporating 4 ml quantities of the solutions at 100° C in a stream of air   The weight of phosphate extracted ($< 0 05$ mg /ml) was negligible compared with the weight of peptide
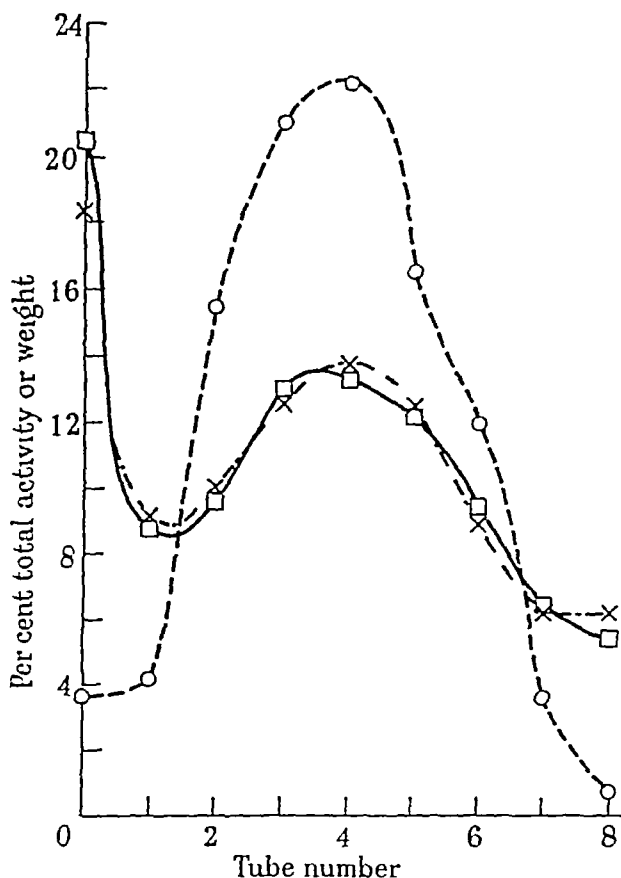
FIG 2 —Eight tube counter current distribution of crude ayfivin (Batch B) between amyl alcohol *n*-butanol (4 1) and m/10 phosphate buffer (pH 6 8)   Lower phase mobile

□————————□ Dry weight   ○ - - - - - ○ Activity
×——.—.—.—× Dry weight estimated by ninhydrin reaction

The amounts of material in the different tubes were also estimated by using the ninhydrin reaction in the manner described by Moore and Stein (1948) Aliquots corresponding to from 20 to 150 μg of material were evaporated to dryness, and the residues hydrolyzed with 0 3 ml amounts of 20 per cent hydrochloric acid in sealed tubes placed in an oven at 110° C for 18 hours   After removal of the hydrochloric acid the ninhydrin colours given by the amino acid mixtures were measured in a photoelectric colorimeter, and compared with those obtained from standard amounts of ayfivin   The method has proved convenient for the estimation of small amounts of material, and has given curves rather

close to those obtained by weighing It will not be reliable if various peptides in a mixture give ninhydrin colours after hydrolysis which differ significantly in intensity

In this distribution the material with the highest specific activity (about 30 units/mg) was found in Tubes 3 and 4 The calculated partition coefficients of the material in the different tubes varied continuously, and no tube appeared to contain a homogeneous substance

Distribution between sec. butanol and 0 5 N acetic acid

The system already described had practical disadvantages Its pH was that at which ayfivin showed a minimum solubility, and several manipulations were necessary to isolate the material in the different fractions, or to obtain it in a form suitable for the determination of dry weight An alternative system was therefore investigated in which the partition coefficient of the activity had a convenient value The two phases were prepared by mutually saturating equal volumes of sec. butyl alcohol and 0 5 N acetic acid The pH of the aqueous phase was about 2 8 The system resembles that used by Barry, Gregory and Craig (1948) for the purification of commercial bacitracin

The partition coefficient of ayfivin in this system expressed as $\dfrac{\text{activity per ml. in sec. butanol}}{\text{activity per ml. in 0 5 N acetic acid}}$ was about 0 5 Fig 3 shows the results obtained from an eight-tube distribution in which the volume of the butanol (4 ml.) was made twice that of the acetic acid (2 ml.) in order to bring the maximum activity near the centre of the series The material used (80 mg.) was part of Batch B, whose distribution curve in the system at pH 7 is shown in Fig 2 At the end of the experiment the amount of material in each tube was determined by evaporation of aliquots from the two phases Activity was measured by the cylinder-plate method after adding 0 1 ml. of the alcohol layer, and 0 05 ml. of the lower layer, to an appropriate volume of water, and extracting the resulting aqueous solution with ether An alternative method, in which all the material was transferred to the aqueous phase by the addition of 12 ml. of ether to each tube, gave almost identical results

Fig 3 shows that the distribution in this system, in contrast to the distribution in the system at pH 7, did not differ greatly from that to be expected if the preparation of ayfivin had consisted of a single substance The fact that the curve rose somewhat more sharply to a maximum than the theoretical curve for homogeneous material could be attributed to small changes in the relative volumes of the phases, which occurred during the transfers Except for Tube 8, which contained a small amount of inactive material, the activity-curve was roughly superimposable on the weight-curve The specific activity of the material isolated from Tubes 3, 4 and 5 was no greater than that of the starting material Very little resolution had therefore occurred under these conditions

Further observations

Although it has been established that the specific activity of crude ayfivin may be raised considerably by eight-tube counter-current distributions at pH 7, the units of activity in the separate fractions have usually appeared to total not

more than 80 per cent of those in the original material   It is thought probable that the loss of activity is partly caused by small amounts of impurities in the solvents used   Inactivation caused in this way becomes more serious the purer the ayfivin and the more dilute the solutions, and has complicated work on the further purification of the material
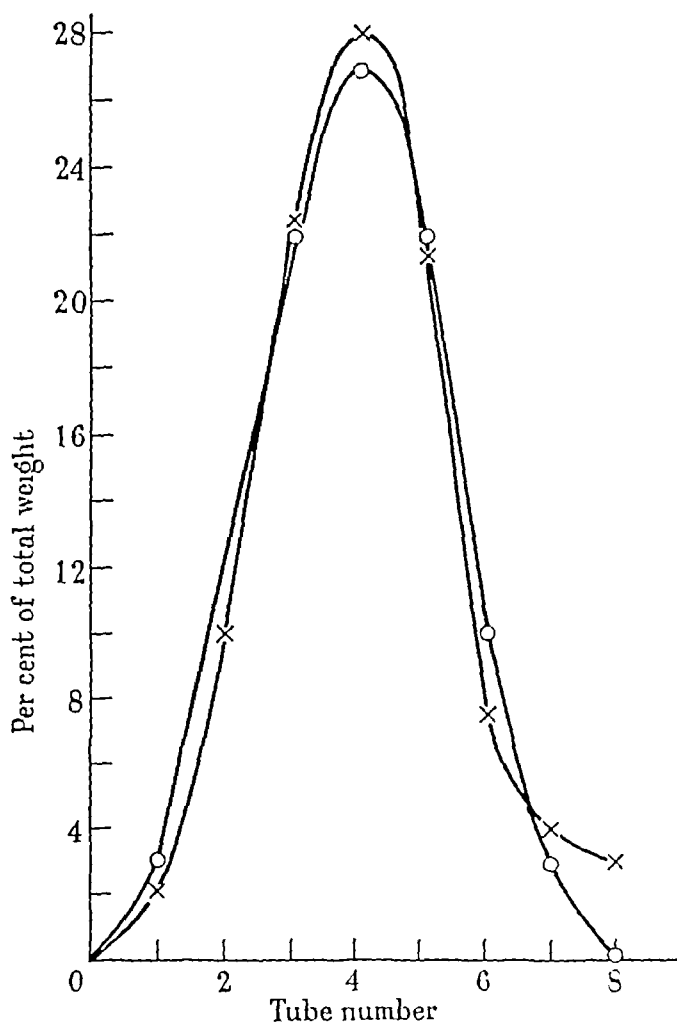
FIG 3 —Eight tube counter current distribution of crude ayfivin (Batch B) between sec butyl alcohol and 0 5 N acetic acid   Lower phase mobile

×————————× Dry weight
O————————O Theoretical curve for a single substance

Physical and Chemical Properties

The physical and chemical properties described here refer to preparations of ayfivin with an activity of about 40 units/mg , which were obtained from Fractions 3, 4, and 5 of eight-tube counter-current distributions at pH 7   These fractions contain the main active constituent of the crude material, but there is evidence that they are not homogeneous   Some of the properties will therefore need re-investigation if pure ayfivin becomes available

Ayfivin hydrochloride gave the following results on elementary analysis

C, 48 8, H, 7 3 , N, 14 6 , S, 1 5 , Cl, 7 9   It contained 2 2 per cent of amino nitrogen according to the van Slyke method of determination   It showed no strong absorption bands in ultraviolet light, but had a weak blue fluorescence It was very soluble in water or ethanol, sparingly soluble in acetone, and insoluble in ether   It dialyzed readily through a cellophane membrane

Free ayfivin was much less soluble in water than the hydrochloride
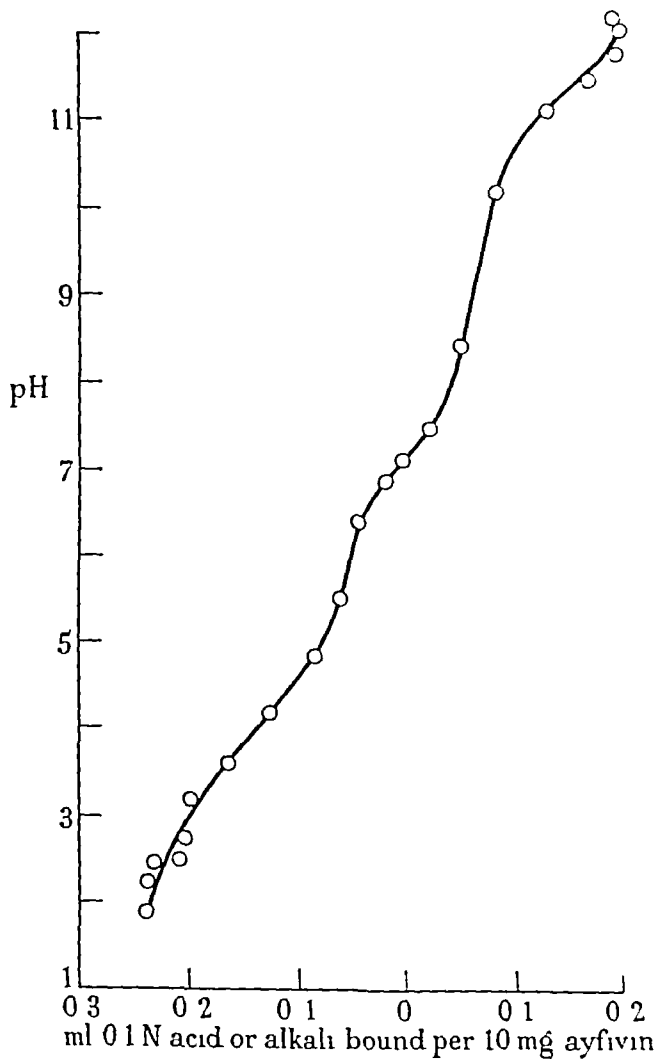
Fig 4 —Titration curve of ayfivin

Ionizable groups

Ayfivin behaved as a weak base   An aqueous solution of its hydrochloride had a pH of about 2 5   Electrometric titration, using a hydrogen electrode, showed that it contained groups ionizing between pH 2 and 5, pH 5 5 and 8 5 and pH 10 and 12 (Fig 4)   Changes in the partition coefficient of the activity between water and *n*-butanol when the pH was varied from 5 5 to 8 5 (see below) showed that ionization in this region was a property of the active material itself The titration curve was compatible with the assumption that ayfivin consisted of

polypeptide   Buffering in the pH range 2–5 could be attributed to the free carboxyl groups of dicarboxylic amino acids , buffering in the pH range 5 5 to 8 5 could be due to the iminazole group of histidine and an σ-amino group , and buffering in the pH range 10 to 12 could be due to free amino groups of diamino monocarboxylic acids   The " isoelectric point " of the material was at pH 7 1

Solubility

Ayfivin was extracted almost completely from aqueous solution between pH 2 and pH 10 by shaking with an equal volume of phenol saturated with water It was not extracted significantly from water at any pH by ether, chloroform, amyl acetate, or benzene
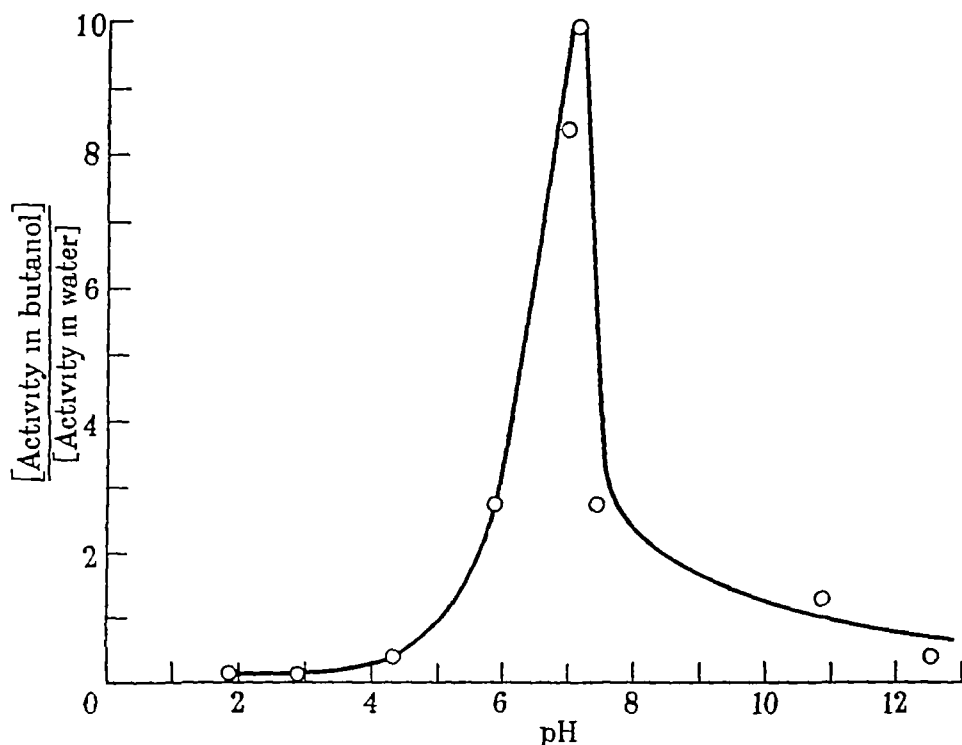
FIG 5 —Variation with pH of the partition coefficient of ayfivin between *n* butanol and water

The partition coefficient of ayfivin (2 mg /ml) between water and *n*-butanol varied greatly with the pH of the aqueous phase   Fig 5 shows the results obtained by measuring the activity in the two phases   The relative solubility in butanol reached a maximum at the isoelectric point (pH 7 1), and fell sharply on either side of this value   The partition coefficient between water and amyl alcohol varied similarly with pH, though ayfivin was considerably less soluble in amyl alcohol than in butyl alcohol

Precipitation and colour reactions

Aqueous solutions of ayfivin gave amorphous precipitates with picric acid, picrolonic acid, Reinecke salt, mercuric chloride, and trichloracetic acid, and also on the addition of saturated ammonium sulphate or sodium chloride   They formed no precipitate with saturated aqueous solutions of benzoic or salicylic acid

When benzoic acid or salicylic acid was precipitated in aqueous solutions of ayfivin very little activity was carried down on the precipitate

Ayfivin gave a positive ninhydrin reaction It gave no coloration with ferric chloride, or with alkaline nitroprusside in the presence or absence of cyanide The Sakaguchi reaction for arginine and the Adamkiewicz reaction for tryptophane were negative Knoop's reaction for histidine was positive

Stability

Ayfivin was stable for 10 minutes when heated at 100° C in aqueous solution (1 mg /ml) at pH 2, 7, or 10 It was stable in 2 N acid for 5 minutes at room
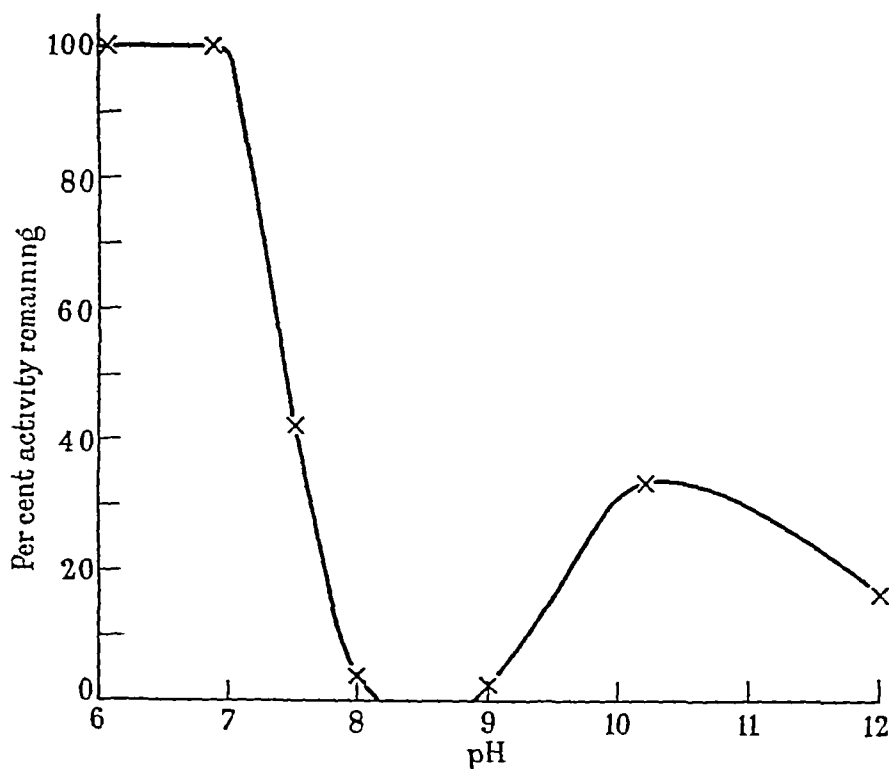
Fig 6 —Inactivation of ayfivin in an aqueous solution (0 1 mg /ml) at different pH values after 4 hours at 37° C

temperature, but was inactivated when heated in 2 N acid at 100° C for 5 minutes It was largely inactivated when kept in N/10 sodium hydroxide for 4 hours at room temperature, or heated in N/100 sodium hydroxide for 15 minutes at 100° C It was not inactivated by incubation with pepsin at pH 3, or with trypsin at pH 8

Inactivation by copper ions

In early experiments with purified ayfivin it was found that material kept at 37° C for 4 hours in dilute aqueous solution (0 1 mg /ml) showed a maximum instability between pH 8 and pH 9 (Fig 6) Between pH 3 and pH 7 no activity was lost, and at pH 10–11 about 70 per cent of the activity was lost while at pH 8–9 the loss of activity was almost complete When dilute solutions of ayfivin at pH 8–9 were heated to 100° C there was almost total loss of activity in 2 minutes

In contrast to the behaviour of purified material, the original culture fluid which had been adjusted to pH 8–9 showed no detectable loss of activity when kept for 4 hours at 37° C , or for 2 minutes at 100° C   The culture fluid therefore contained sustances that protected ayfivin from inactivation   It was found that the inactivation of purified ayfivin at pH 8–9 was largely or completely prevented by the following additions to the solution   commercial trypsin or pepsin (1 mg /ml) , horse serum (1 per cent) , heart or lemco broth , glutamic acid, *cysteine, or glycine (1 mg /ml)   Gelatine (1 mg /ml) exerted some protective action*, but was less effective than the other substances

When the concentration of ayfivin was increased from 0 1 mg /ml to 1 mg /ml very little loss of activity was detected on keeping the solution at pH 8–9 for 4 hours at 37° C   The change in apparent stability with concentration suggested that the inactivation was caused by traces of an impurity in the solvent (laboratory distilled water)   If the amount of ayfivin inactivated depended largely on the amount of the impurity, and thus on the volume of the solvent, the *proportion* of ayfivin inactivated would diminish as its concentration was increased

It is now considered that a reaction of ayfivin with copper ions was responsible for this phenomenon   When the early experiments were later repeated no inactivation was found to occur   In the interval a new apparatus had been installed to supply distilled water   Inactivation occurred at pH 8–9 in the new distilled water, but not at pH 7 or below, when small amounts of copper sulphate were added to the solution   Table I shows the results obtained when ayfivin (0 1 mg /ml) was incubated for 4 hours at pH 8 4 with varying amounts of cupric ions

An amount of Cu^{++} as small as 0 25 µg /ml caused substantial inactivation Little, if any, inactivation was caused under similar conditions by a number of other heavy metal ions in concentrations approximately 10 times as large   The ions tested were Pb^{++}, Fe^{+++}, Zn^{++}, Co^{++}, Mn^{++}, Al^{+++}, Sn^{++}, and Hg^{++} The loss of activity, which occurred in the presence of 2 5 µg Cu^{++} per ml , was prevented by the addition of glutamic acid (1 mg /ml) to the solution

Attempts to restore the activity of ayfivin inactivated in the presence of cupric ions have not so far been successful

TABLE I

Cu^{++} µg /ml	Per cent of activity remaining
25 0	0
2 5	0
0 25	10
0 025	90
0 0025	100
0 00	100

Amino acids formed on acid hydrolysis

When ayfivin was hydrolyzed for 24 hours with boiling 20 per cent hydrochloric acid almost all of its nitrogen appeared as α-amino nitrogen   The product showed a much stronger ninhydrin reaction than the original substance   When the ninhydrin reaction was carried out in the manner described by Moore and Stein (1948) the intensity of the colour shown by the hydrolysate from a given

weight of ayfivin hydrochloride was very close to that shown by the same weight of leucine   The hydrolysate gave a positive test for SH with nitroprusside in the presence, but not in the absence, of sodium cyanide

Paper chromatography

Using the method introduced by Consden, Gordon and Martin (1944), one-dimensional chromatograms on Whatman No 1 paper were obtained from the ayfivin hydrolysate with the phenol-water system in the presence of HCN, with the phenol-water system in the presence of ammonia, and with the benzyl alcohol-water system   Chromatograms were also obtained from the acidic and from the basic plus neutral fractions of the hydrolysate which had been separated on Amberlite IR-4 by the method of Consden, Gordon and Martin (1948)   The positions of the spots after development with ninhydrin indicated that the following amino acids were present   aspartic acid, glutamic acid, lysine, ornithine, histidine, cystine, phenylalanine, leucine and/or isoleucine   The amount of the leucines appeared to be larger than that of any other single amino acid

Oxidation with D-amino acid oxidase

The oxygen absorbed on treating ayfivin hydrolysate with a preparation of D-amino acid oxidase (Krebs, 1935) at 37° C was measured in the Warburg manometric apparatus   The activity of the enzyme system was tested with DL-alanine as a substrate   the reaction was complete in 1 hour, and the oxygen taken up corresponded to the theoretical amount for the oxidation of the D-component   With ayfivin hydrolysate as a substrate the reaction appeared to be complete in 2 hours, and the amount of oxygen absorbed corresponded to 14 c mm per mg of ayfivin hydrochloride   On this basis about 14 per cent of the nitrogen of the ayfivin hydrolysate was in the form of D-α-amino nitrogen

DISCUSSION

There can be little doubt that the main active constituent of ayfivin is a highly polar polypeptide   Although there is evidence that the most active preparations described in this paper are not homogeneous, their impurities appear to have physico-chemical properties and to contain amino acid radicals similar to those of the antibiotic itself   Even in the case of the crude product, the solubilities of the impurities were such that very little resolution occurred during an eight-tube counter-current distribution under acid conditions, and the material behaved as though it were largely a single compound   The fact that this material was quickly resolved into several components in a system at pH 7, and that some of the resulting fractions showed a considerable increase in specific activity, indicates that conclusions about the homogeneity of a polypeptide which are based on results from a restricted number of distributions in one system can easily be too optimistic   Craig, Mighton, Titus and Golumbic (1948) have pointed out that the results of counter-current distributions, like those of all other methods, can only provide an answer to the question of the purity of a substance in terms of probability   With relatively large molecules several different criteria are desirable before the probability of a substance being homogeneous is considered to be very high

The variation in the partition coefficient of ayfivin between butanol and water with changes in pH indicates that the active substance contains groups

that ionize over a wide range of hydrogen ion concentrations   The presence of such groups in the purified material is shown by the results of electrometric titration and of qualitative amino acid analyses   Anionic centres are provided by aspartic acid and glutamic acid, and cationic centres by histidine, ornithine and lysin   The hydrophobic side-chains of leucine and phenylalanine, which appear to be the main neutral amino-acids, would account in part for the high solubility of ayfivin in moist *n*-butanol at the isoelectric point   At this point the solubility may be increased by a displacement of equilibrium from the polypolar towards the non-charged form

The electrometric titration of the purest preparation reveals a group which titrates in the region immediately on the alkaline side of the isoelectric point   A group in ayfivin, which ionizes in this region, may be concerned with the inactivation of the antibiotic that occurs under certain conditions in the presence of copper ions   The amount of inactivation is negligible at pH 7, but rapidly reaches a maximum at pH 8 5   A copper co-ordination complex is possibly involved in the reaction   The fact that there is a decrease in the amount of inactivation when the pH is raised from 8 5 to 10 would be understandable if a group inessential for antibacterial activity, either in ayfivin itself or in impurities, competed for copper at the higher pH   Competition by added amino acids and proteins has been shown to prevent inactivation

An antibiotic named licheniformin, which is produced by a strain of *B licheniformis*, has been studied by Callow, Glover and Hart (1947), and by Callow, Glover, Hart and Hills (1947)   When grown under appropriate conditions, the strain A-5 of *B licheniformis* also produces a licheniformin-like antibiotic (Hills *et al*, 1949)   Ayfivin and licheniformin differ markedly in chemical properties   Ayfivin, however, shows similarities to the antibiotic bacitracin, first described by Johnson, Anker and Meleney (1945)   When the present work began little published information on the chemical properties of bacitracin was available, and it was doubtful whether the substance was a peptide (Anker, Johnson, Goldberg and Meleney, 1948)   Both ayfivin and bacitracin could be extracted from culture fluid with *n*-butanol, but it was thought unlikely that they were identical because bacitracin was reported by Johnson, Anker, Scudi and Goldberg (1947) to be precipitated by benzoic acid and salicylic acid, and these reagents did not remove ayfivin from solution   A sample of commercial bacitracin, however, has now been found to behave like ayfivin towards benzoic and salicylic acid   The work of Barry, Gregory and Craig (1948) has established that purified preparations of bacitracin consist of polypeptide, and the amino acids they are reported to contain are very similar to the amino acids present in the most active preparations of ayfivin   Further investigations are required to establish the exact relationship between the two antibiotics

Since this paper was written it has been shown that the inactivation in the presence of copper ions is accompanied by an uptake of oxygen and that it probably involves the oxidation of a suphur atom

SUMMARY

1   Preparations of ayfivin with high antibacterial activity were obtained from the culture fluid of the strain A-5 of *B licheniformis*   The process of extraction involved adsorption of the active material on to charcoal, elution with a mixture

of n-butanol and dilute hydrochloric acid, transfer between butanol and water, and precipitation with picric acid The crude product was further purified by 8-tube counter-current distributions in a system composed of amyl alcohol, n-butanol, and water or phosphate buffer at pH 7 No significant purification was obtained by 8-tube distributions in a system containing acetic acid at about pH 2 8

2 The most active material behaved as a polypeptide with an isoelectric point at pH 7 1   It contained groups which ionized in the pH region 2–5, 5 5–8 5, and 10–12   On acid hydrolysis it yielded aspartic acid, glutamic acid, histidine, ornithine, lysine, cystine, phenylalanine, leucine and/or isoleucine

3 The partition coefficient (ayfivin in n-butanol)/(ayfivin in water) varied rapidly with the pH in region of neutrality and was a maximum at the isoelectric point   On the alkaline side of the isoelectric point ayfivin was inactivated in the presence of copper ions, the amount of inactivation reaching a maximum at pH 8–9

4 Ayfivin is quite different from licheniformin in its physical and chemical properties, but shows a similarity to bacitracin

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BIOLOGICAL OBSERVATIONS ON AYFIVIN

A ARRIAGADA, H W FLOREY, M A JENNINGS AND I G WALLMARK

From the Sir William Dunn School of Pathology, Oxford

THE following paper records observations on the antibacterial, pharmacological and chemotherapeutic properties of impure ayfivin, the preparation and chemical properties of which have been described by Sharp, Arriagada, Newton and Abraham (1949)

EXPERIMENTAL

Antibacterial Properties

The activity of ayfivin against a number of species of bacteria was tested by the serial dilution method   Glass-filtered solutions of a crude preparation containing about 10 units/mg were used for most of the experiments   This solution was diluted in a two-fold series and added in the proportion of 1 in 10 to tubes of heart extract broth, which were then inoculated with a uniform amount of culture of the organism under test   According to the hardiness of the organism the culture was used undiluted, or was diluted 1 in 10, 1 in 100, or 1 in 1000, so as to give a small inoculum, yet one that would be certain to grow   Modifications of the technique were made when necessary, for instance, 10 per cent serum was incorporated for the neisseria and some of the streptococci, and the tubercle bacillus was tested in Dubos' Tween and albumen medium   The clostridia were incubated in anaerobic jars

Table I shows the results, in most cases after overnight incubation   Incubation for a further 24 hours had usually no effect on the titre, but occasionally doubled the concentration needed for complete inhibition

Nine of the strains of *Staph aureus* that were tested were relatively resistant to penicillin, requiring from 8 to 200 units/ml for inhibition   These had the same sensitivity to ayfivin as the penicillin-sensitive strains

The effect of ayfivin on two strains of *Leptospira icterohaemorrhagiae* in 12 per cent serum water was tested by a similar method *   In tests *in vitro* with this organism death rather than inhibition of multiplication is observed   Both strains were killed by a concentration of 1 in 10,000, higher dilutions having little or no effect

Bactericidal action

Dilution series that had been inoculated with *Str pyogenes* and two strains of *Micrococcus* were plated out after 20 hours' incubation   There was no growth from the tubes containing the strongest concentrations, the smallest concen-

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* We are indebted to Dr J A H Wylie for carrying out these tests

tiation that had killed ranged from the same as that which had inhibited growth to one 4 times greater

There was no evidence that *Myco tuberculosis* was killed by ayfivin

TABLE I—*Antibacterial Activity of Ayfivin The figures in the third column show the concentrations of ayfivin in units/ml which completely inhibited growth in liquid medium during 20 hours' incubation (44 hours in the case of the* Clostridia *and longer periods for* Myco tuberculosis*)*

Species	Number of strains	Concentration inhibiting growth (units/ml)
Staphylococcus aureus	18	0 125–>1 0
,, *albus*	1	1 0
Streptococcus pyogenes		
Group A	5	0 015–0 063
,, B	1	>1 0
,, D and *Str faecalis*	3	>1 0
,, G	1	0 5
Streptococcus viridans	5	0 25–1 0
,, *pneumoniae*, Types 1, 3, 6, 8, 19, and 2 strains not typed	8	0 031–0 5
Micrococcus lysodeikticus	1	0 031
,, *tetragenus*	1	1 0
,, sp	1	0 015
Neisseria gonorrhoeae	1	0 25
,, *meningitidis*	2	0 5, 1 0
Corynebacterium diphtheriae var *gravis, intermedius* and *mitis*	3	0 063–>1 0
Corynebacterium xerosis	1	0 008
Clostridium welchii	2	>1 0
,, *septicum*	1	>1 0
,, *oedematiens*	1	>1 0
,, *novyi*	2	>1 0
Bacillus anthracis	6	>1 0
Mycobacterium phlei	2	>1 0
,, *tuberculosis*,* bovine type	2	>2 0
,, ,, * human type	8	0 5–72
,, ,, * B C G	1	0 5
Haemophilus influenzae	3	>1 0
,, *parainfluenzae*	2	>1 0
Pasteurella pestis	1	>1 0
,, sp	1	>1 0
Salmonella typhi	2	>1 0
,, *enteritidis*	3	>1 0
Bacterium coli	2	>1 0
Pseudomonas pyocyanea	1	>1 0
Proteus	2	>1 0
Vibrio cholerae	2	>1 0

* We are indebted to Dr A. Q Wells for these determinations

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Effect of inoculum size

With both *Staph aureus* and *Str pyogenes* alterations in the size of the inoculum had little effect on the titre, the greatest difference being a four-fold increase in the inhibiting concentration as the result of a thousand-fold increase in the size of the inoculum

Effect of serum

The presence of 50 per cent of serum had little or no effect on the activity of ayfivin In liquid culture medium it increased the concentration needed to inhibit the growth of *Str pyogenes* from 2 to 4 times, but this difference may have been associated with stronger growth of the organism in the enriched medium

Solutions of ayfivin were also incubated for 4 hours with 50 per cent serum, and then diluted serially and tested on cylinder plates alongside a control solution that had been incubated with water The plates were seeded with *C xerosis* There was no alteration in the size of the zone of inhibition as the result of the presence of serum

Acquisition of resistance

In vitro —Using material containing 40–45 units/mg , *Staph aureus* (National Collection of Type Cultures, No 6571) was transferred from one dilution series to another 23 times in 6 weeks Each transfer was made from the tube containing the highest concentration of ayfivin in the presence of which there was substantial growth There was no increase of resistance at any time during the experiment

Myco tuberculosis, however, developed resistance to ayfivin after growth in its presence *in vitro* *

In vivo —Cultures of *Staph aureus* recovered from mice that had been infected with that organism and treated with ayfivin for 4 days, the last dose being given from 3 to 10 days previously, showed no sign of increased resistance The same was found after therapeutic experiments with *Str pyogenes*

Comparison of Ayfivin with Bacitracin by Serial Dilution Tests

Towards the end of the work reported in this paper it became apparent that ayfivin was very similar to bacitracin, if not identical with it There were some discrepancies between the reported sensitivity of certain anaerobic spore-forming organisms and Group B *Streptococcus* to bacitracin and those found for ayfivin A direct comparison between ayfivin and bacitracin (kindly supplied by Dr Meleney) by the serial dilution method already outlined disclosed no essential difference between the sensitivities of one strain each of *Cl welchii*, *Cl sporogenes*, *Streptococcus* Group A and *Streptococcus* Group B to bacitracin and ayfivin

PHARMACOLOGICAL OBSERVATIONS
Toxicity

Acute toxicity to mice

A comparison was made between the toxicity of single doses of two fractions obtained by the counter-current distribution method of Craig (1944)   One

* For this observation we are indebted to Dr A Q Wells

fraction contained 30 units/mg and the other 4 units/mg Table II shows the mortality after intravenous injection into mice weighing 19 to 20 g

TABLE II —*Effect of Intravenous Injection of Two Fractions of Ayfivin*

Units/mg in sample	Dose in 0 3 ml saline		Total number of mice	Number of mice alive at the end of day—					
	Mg	Units		1	2	3	4	5	29
30	2 5	75 0	10	10	10	10	10	10	10
30	5 0	150 0	10	4	4	4	4	4	4
4	5 0	20 0	10	10	10	10	10	10	10
30	7 5	225 0	10	5	4	3	3	2	2
4	10	40 0	10	5	2	0	0	0	0

Table III shows the mortality after the subcutaneous injection of the same samples of ayfivin into mice weighing 20 g It is apparent from these results that the fraction containing more ayfivin was more toxic than the less antibacterially potent fraction, which nevertheless contained toxic substances that were bacteriologically inert

TABLE III —*Effect of Subcutaneous Injection of Two Fractions of Ayfivin*

Units/mg in sample	Dose in 0 6 ml saline		Total number of mice	Number of mice alive at the end of day—			
	Mg	Units		1	2	3	29
30	10	300	10	10	7	5	5
4	10	40	10	10	10	10	10
30	20	600	10	10	1	1	1
4	20	80	10	9	4	2	2

The animals that died in a short time showed inco-ordinated movements before reaching a state of collapse

Chronic toxicity to mice

Mice weighing initially about 20 g were injected with material containing 29 units per mg Five mice received 5 mg and 5 mice 10 mg daily, divided into 4 doses, and given subcutaneously (a total of 1305 and 2610 units per mouse respectively) for 9 days The animals gained in weight in the same way as control animals receiving subcutaneous injections of normal saline, and showed no sign of toxic reactions

In another experiment material containing 32 units per mg was used Mice in 3 groups of 4 were given total doses of 48 mg (1536 units), 24 mg (768 units) and 12 mg (384 units) respectively by subcutaneous injection in 6 days, four equal doses being given daily In the group receiving the largest dose one mouse died and another became sick but recovered The rest of the mice apparently suffered no ill effects from the drug

In a therapeutic experiment to be described later 5 mice which received 40 mg (1280 units) of similar material (32 units/mg) in 4 days were in good condition at the end of 6 weeks

Effect on the kidneys

Histological evidence suggested that the death of mice receiving the larger repeated doses of ayfivin might be due at least in part to damage to the kidneys Other organs appeared to be normal, but the condition of the kidneys was as follows

After single intravenous doses of 5–7 5 mg (of material containing 32 units/mg) no kidney lesions were found when the animals were killed 3 and 17 days later

After multiple small subcutaneous doses —Two doses of 4 mg (of the same material) were given subcutaneously on one day The mouse was killed 3 days later The epithelium of some of the tubules was much flattened, and the tubules throughout the kidney were filled with hyaline masses There was little evidence of necrosis, but there were many mitoses of the tubule epithelium

Another mouse received 5 doses of 4 mg (of the same material) in 2 days, and was found dead 17 hours after the last dose Here the hyaline material in places contained dead cells, and there was evidence of degeneration or necrosis of much of the epithelium A mouse which received 9 consecutive doses of the same amount in 4 days survived, and was killed 17 days later At this time the epithelium showed little sign of damage, but here and there in the superficial part of the cortex were small areas of activity where regeneration of the epithelium appeared to be in progress Tubules in some of these areas were dilated to form small cysts

After longer courses of smaller injections—2, 4 or 8 mg of the same material daily for 6 days, 12, 24 or 48 mg in all—there was no consistent evidence of damage, though kidneys from mice that were killed a few days after the last injection usually showed a few of the areas of regeneration described above

In the therapeutic experiment to be mentioned, in which each of 5 mice received a total of 40 mg , the kidneys appeared to be normal when the animals were killed after 6 weeks

There is no doubt that, like other antibacterial polypeptides, impure ayfivin in sufficient dosage sometimes severely damages the kidneys of mice, though the presence of lesions was not closely related to the number of units given

Local effects on tissues

No damage was caused to the skin of mice by the subcutaneous injection of solutions of ayfivin (30 units/mg) containing 16 6 mg /ml Five mice, given 2 0 mg of material containing 32 units/mg 5 times a day for 4 days in some of the therapeutic experiments to be described, showed no sign of damage in the skin or subcutaneous tissues when killed 6 weeks after the last injection, nor was damage to the skin noted in those dying during the therapeutic experiments

Effect on the motility of human leucocytes and on red blood cells

Human leucocytes were examined by the method of Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941) No deviation from control preparations was observed when the leucocytes were immersed in a concentration of 1 in 1000 ayfivin , in a concentration of 1 in 500 all leucocytes appeared to be active at the end of $2\frac{1}{2}$ hours, but thereafter they became more sluggish than

those in control preparations   In a concentration of 1 in 250 cells were active
for about an hour, but thereafter became immobile
The red blood cells in the preparations were unaffected by any of the above
concentrations

Respiration

Blood
pressure
100 mm Hg

Time
10 seconds
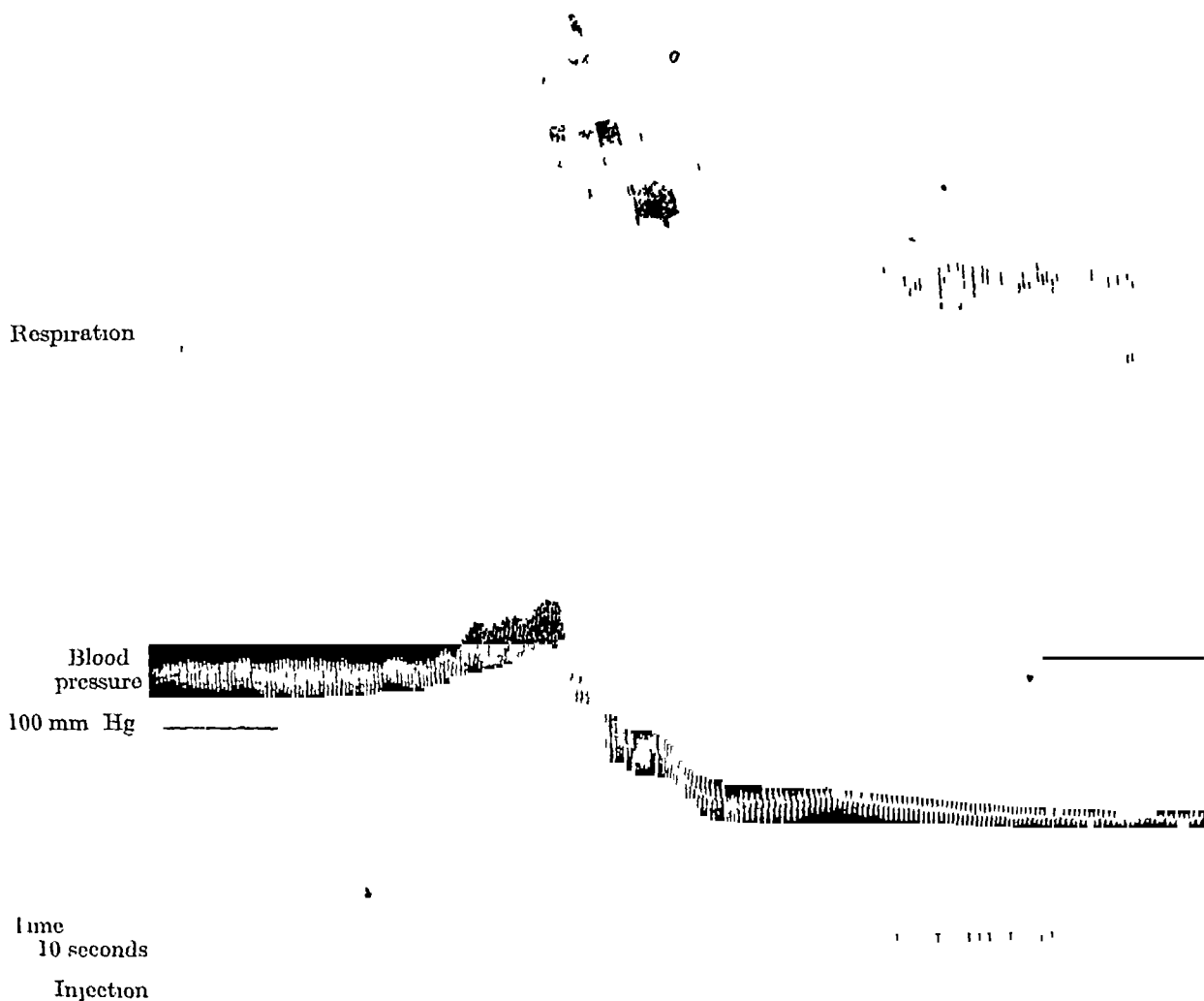
Injection

Fig 1 —The effects on respiration and blood pressure of injecting 217 5 mg of ayfivin intravenously
into a decerebrate cat weighing 2 9 kg

Effect on blood pressure and respiration

Four experiments were made on cats anaesthetized with chloralose   The
injection of 50 mg of material containing 30 units per mg into a cat weighing
3 kg caused no fall of blood pressure   a further injection of 100 mg 14 minutes
later caused a slow fall from 134 to 126 mm Hg , a further injection of 200 mg
caused a fall from 126 mm to 72 mm Hg in 14 minutes   The pressure gradually

returned to 126 mm Hg during the next 2 hours   In the other animals similar results were obtained with smaller doses of less pure material

Experiments were also done with cats decerebrated according to the method of Schmidt (1923)   Fig 1 illustrates the result of injecting a cat weighing 2 9 kg with 75 mg /kg (217 5 mg) intravenously   There was a profound fall of blood pressure and stimulation of respiration   No recovery in the level of the blood pressure occurred   Doses of 25 and 50 mg given intravenously to other decerebrate cats had no effect on the respiration or blood pressure

It is clear that if a sufficiently large dose is given the substance causes a substantial fall of blood pressure

Effect on smooth muscle

The effects of ayfivin on smooth muscle were tested on guinea-pig uterus suspended in oxygenated Ringer-Locke's solution   A concentration of 1 in 5000 ayfivin (material containing 30 units/mg) sometimes increased the amplitude of the rhythmic contractions , the effect was more marked with a concentration of 1 in 1250   After washing, the amplitude of the contractions became less again (Fig 2)   Sometimes no definite effect was observed

Thus it is unlikely that the fall of blood pressure following intravenous injection into the cat is due to the presence of histamine-like substances in preparations of ayfivin

Absorption and Excretion

Absorption and excretion in mice

It was found that ayfivin was absorbed after subcutaneous injection in mice and appeared in the urine   Use was made of its excretion by the kidneys to determine whether there was significant absorption from the alimentary canal

Urine was collected from 2 mice weighing 20 g 5 hours after the administration of 320 and 160 units by stomach-tube   The stomach and intestines were washed out with buffer solution and the washings were assayed For comparison, similar observations were made on 2 mice that had received 32 units subcutaneously   Specimens were assayed by the cylinder-plate method, using *C xerosis* as the test organism   Table IV records the results

TABLE IV

Mouse No	Route	Dose, units	Total amount (units) recovered from	
			urine	gastro intes tinal tract
1	*Per os*	320	0	60
2	,,	320	0 45	27
3	,,	160	0 2	10 5
4	,,	160	0	13 5
5	Subcutaneous	32	16 5	0
6	,,	32	3	0 2

It is clear that little or no ayfivin is excreted in the urine following oral administration, and it may be inferred that the substance is not absorbed in significant amounts from the alimentary tract

r 5,000

FIG 2A

l 1250
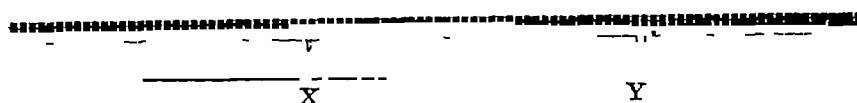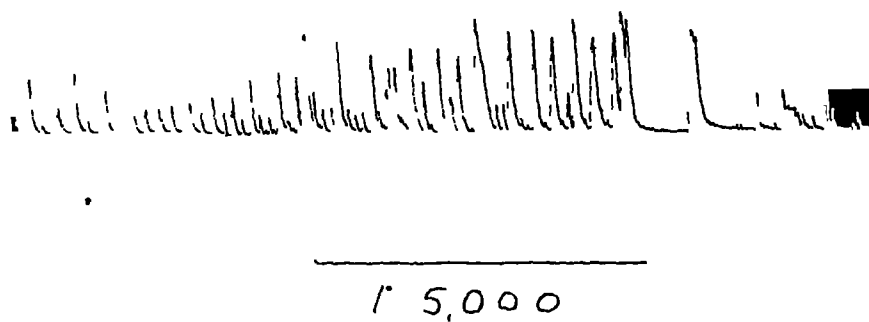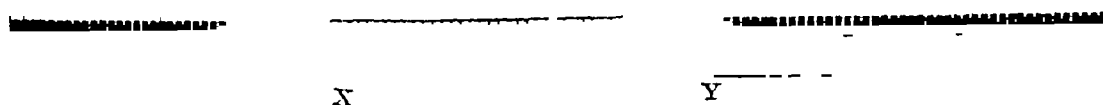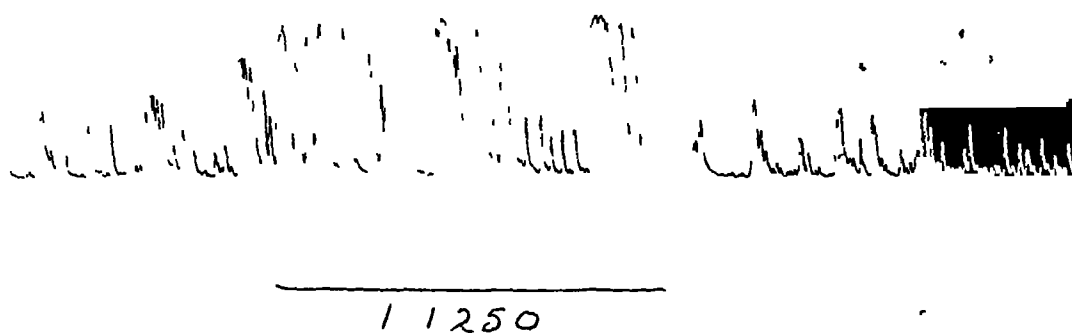
FIG 2B

Fig 2 — A At x, ayfivin added to bath to make a concentration of 1 in 5000   At y, uterus washed with Ringer's solution   Time   30 seconds   B Similar to A, but concentrations 1 in 1250

Blood levels and urinary excretion in rabbits

Rabbits were injected subcutaneously with a sterile solution of ayfivin, and blood samples were withdrawn at intervals afterwards   Assays were performed on the serum by the micro-method described by Fleming (1944), using *Str pyogenes* as test organism and red blood cells as indicator   The urine was collected in a metabolism cage and assayed by the cylinder-plate method, using *C xerosis* as the test organism   Some of the results are shown in Fig 3

Animal Protection Experiments

Against Str pyogenes

In 1947 the first experiments were done, using two groups of 10 mice which were infected by the intraperitoneal injection of 0 5 ml of a 1 in 100 dilution of an overnight culture of the organism
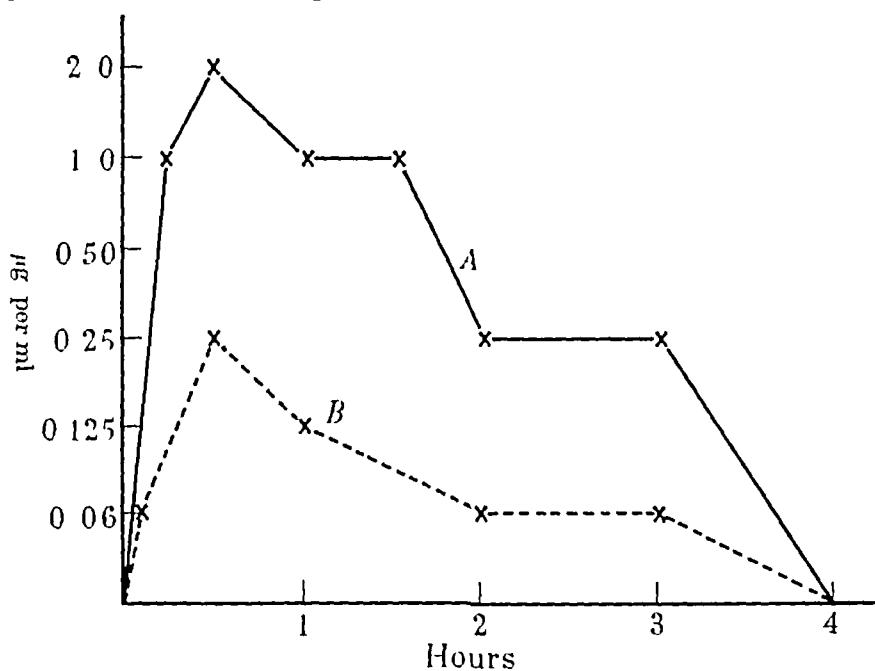
Fig 3 —Concentrations of ayfivin in the blood serum of two rabbits after subcutaneous injec
tion   A ——— 30 mg (960 units) injected   B ———   10 mg (320 units) injected
Total recovered in urine   A 575 units, i e 60 per cent of amount injected   B 173 units,
i e 45 per cent of amount injected.

A sample of ayfivin containing 10 units/mg was used   In the first experiment a total of 9 5 mg (95 units) was administered in divided doses 4 times daily for 2 days   The 10 control mice died within 36 hours, while the 10 treated mice were alive and well at the end of 10 days   In two other experiments conducted at this time it was found that even after as little as 0 5 mg (5 units) given in 48 hours life was prolonged in all treated animals, and 1 mouse out of 5 was alive at the end of 8 days

A more thorough repetition of these experiments was made in 1949, when it was again demonstrated that there was a chemotherapeutic effect (see Table V) *Str pyogenes* (Wellcome CN 10), of which the lethal dose for mice was about 500 organisms, was used for infection   Its growth was inhibited *in vitro* by one part

TABLE V —*Action of Ayfivin in Mice Infected with* Str pyogenes

Dose of ayfivin (units)		Total number of mice	Number of mice alive at end of day—							
Single	Total (in 2 days)		1	2	3	4	5	6	7	31
15	150	5	—	—	—	—	—	—	5	5
7 5	75	5	—	5	4	3	—	—	3	3
3 75	37 5	5	—	5	3	2	—	—	2	2
1 88	18 75	5	—	5	2	—	—	—	1	1
0 95	9 5	5	5	2	1	—	—	—	1	1
No treatment (controls)		20	2	0						

of this preparation of ayfivin in 2 56 millions, that is by 0 012 units per ml Mice weighing from 22 to 24 g were injected intraperitoneally with 0 5 ml of a 1 in 10,000 dilution of an overnight culture containing about 50,000 bacteria—that is, 100 lethal doses Five groups of 5 mice each were treated with ayfivin given subcutaneously, and 20 untreated mice formed a control group The preparation used contained 32 units per mg On two successive days four injections were made at approximately 4-hour intervals, the first three injections on each day containing from 0 95 to 15 units for different groups, and the last containing a double dose Even the smallest dose had some effect in prolonging life, but only the largest dose (150 units in all) gave complete protection Streptococci were recovered from the heart blood of the animals dying during the experiment The mice that survived gained weight, and when they were killed at 31 days postmortem examination showed nothing abnormal

Against Staph aureus

In 1947 experiments were performed with mice infected intraperitoneally with 1 ml of a 1 in 5000 dilution of an overnight broth culture of *Staph aureus* in 5 per cent mucin Ayfivin containing 28 units per mg was used for treatment In the most successful experiment all of 7 treated mice survived in good condition for 1 month, after which they were not followed further These mice received 3 doses of 2 mg and 1 of 4 mg daily, a total of 40 mg (1120 units) being given in 4 days In the same experiment mice receiving half this amount began to die on the 7th day, and gradually all died with localized staphylococcal abscesses

In 1949 this investigation was repeated in more detail (Table VI) In Experiment 1, 45 mice weighing from 21 to 23 g were used An overnight serum broth culture of *Staph aureus* (Wellcome CN 491), suspended in 5 per cent mucin to give a final dilution of the culture of 1 in 1000, was used to infect 45 mice, 0 5 ml being injected intraperitoneally into each animal This was equivalent to about 10 lethal doses The plan of the experiment followed that for *Str pyogenes*, and the same preparation of ayfivin was used but larger doses were given the staphylococcus being relatively more resistant by in vitro tests The growth of strain CN 491 was inhibited by a concentration of 1 part of this preparation in 160,000, that is by 0 2 unit/ml

Single doses ranged from 4 to 64 units for different groups the spacing of the doses and the double dose in the evening being the same as for the streptococcus

The earlier experiments had shown how hard it was to eradicate the staphylococcus completely, so treatment was continued for 4 days

The 20 control mice were all dead with staphylococcal septicaemia within 36 hours   In the group of mice that received a total dose of 320 units two mice were killed by their companions between the 17th and 21st days   These showed no sign of staphylococcal infection, and the heart blood was sterile   All surviving treated mice were killed at the end of 6 weeks, at which time they appeared to be in good health, and had gained weight   On post-mortem examination 3 of the 5 mice that had received the smallest dose (total 80 units) were found to have staphylococcal abscesses in the liver and peritoneal cavity

Table VI shows also the dosages and results in two subsequent experiments carried out in a similar way   In Experiment 2 the dose of staphylococcus culture in mucin was the same, but the organism had lost virulence, and the control animals were not all dead until the 15th day   In spite of the lower virulence, perhaps because the number of organisms used was the same, there was no reduction in the total dose of ayfivin needed to save life   By the time that Experiment 3 was performed the strain had recovered virulence about equal to that in Experiment 1   Ten times as many bacteria were injected (about 100 lethal doses), again in 5 per cent mucin, and this killed all control mice within 14 hours   Protection by the smaller doses of ayfivin was not so effective in this group

Treated mice that survived for 6 weeks showed no sign of ill-health, and gained weight, but out of the total of 41 that survived and were killed at this time 8 had staphylococcal abscesses in the abdominal wall, in the mesentery, or

TABLE VI —*Action of Ayfivin in Mice Infected with* Staph aureus

Experiment No	Dose of culture diluted in 5 per cent mucin[1]	Dose of ayfivin (units) Single	Total (in 4 days)	Number of mice, total	Day 1	2	3	4	5	6	7	Week 2	3	4	5	6
1	0 5 ml of 1 1000	64	1280	5	—	—	—	—	—	—	5	—	—	—	—	5
		32	640	5	—	—	—	—	—	—	5	—	—	—	—	5
		16	320	5	—	—	—	—	—	—	5	4[2]	3[2]	—	—	3
		8	160	5	—	—	—	—	—	—	5	—	—	—	—	5
		4	80	5	—	—	—	—	—	—	5	—	—	—	—	5[3]
	No treatment (controls)			20	3	0										
2	0 5 ml of 1 1000	13	256	5	—	—	—	—	—	—	5	—	—	—	—	5[4]
		6 5	128	5	—	—	—	—	—	—	5	—	—	4	—	4[5]
		3 3	64	5	—	—	—	—	—	—	5	—	2	—	—	2
		1 6	32	5	—	—	—	—	—	—	5	3	2	—	—	2
		0 8	16	5	4	3	—	—	—	—	3	2	1	—	—	1[5]
	No treatment (controls)			10	6	5	—	—	3	—	3	1	0*			
3	0 5 ml of 1 100	16	320	5	—	—	—	—	—	—	5	—	4	—	—	4[5]
		8	160	5	—	—	—	—	—	—	5	3	0			
		4	80	5	—	4	—	—	—	—	4	2	0			
		2	40	5	4	3	—	1	0							
		1	20	5	3	1	—	0								
	No treatment (controls)			10	0†											

[1] Virulence of staphylococcus fell at time of Experiment 2, and recovered afterwards
[2] Killed by fighting
[3] Three survivors had staphylococcal abscesses
[4] Two survivors had staphylococcal abscesses
[5] One survivor had staphylococcal abscesses
* Last control mouse died on 15th day
† All control mice dead within 14 hours

in the liver It was thus evident that the doses used did not eliminate staphylococci completely, except possibly in Experiment 1, in which the largest dose was 4 times as great as any dose used subsequently, and was near the toxic level

Thus ayfivin, when administered in the proper dosage, can protect mice against certainly fatal infection with *Str pyogenes* and *Staph aureus*

DISCUSSION

It is clear from the data presented that ayfivin is one of those polypeptides which, under appropriate conditions, are capable of exerting a systemic therapeutic effect in mice, but which have the great disadvantage of causing severe damage to the kidneys in doses not much above those necessary for therapeutic effect

The pharmacological properties and antibacterial range of ayfivin are very similar to those of bacitracin During most of the time that this work was going on it was thought that there were differences between the two crude products, for some of the chemical and biological data originally published on bacitracin did not appear to accord with those on ayfivin, but a comparison recently made between crude ayfivin and crude bacitracin (such as is used for clinical work) has not so far revealed any chemical difference (Sharp *et al*, 1949), nor has any biological difference been discovered In the comparative antibacterial test reported here their range of activity was precisely the same It is quite possible that ayfivin and bacitracin, though similar, are not identical, but this point may not be settled until the active polypeptides can be compared in a much more highly purified form

SUMMARY

Ayfivin, an impure polypeptide mixture obtained from a strain of *B licheniformis*, has been shown to have powerful antibacterial properties and a low toxicity to mice It has been shown to possess protective action against experimental infections in mice with *Str pyogenes* and *Staph aureus* However, in doses not much greater than those required for therapeutic effect severe damage can be caused to the kidneys Ayfivin appears to be closely related to bacitracin

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THE PREPARATION AND PROPERTIES OF ENZYMES FROM *CL WELCHII* (TYPE B) FILTRATES WHICH DESTROY BLOOD-GROUP SUBSTANCES

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SCHIFF (1935, 1939) first observed that cultures and culture filtrates from certain strains of *Clostridium welchii* possess the power to destroy the specific serological properties of the blood-group A-substance present in commercial peptone and human saliva   Schiff considered the enzyme to be specific for the A-substance and to be without action on the Group B factor in saliva

Schiff and Weiler (1931*a*) observed that the normal faeces of persons of all groups and of certain animals contain an enzyme which readily inactivates the A and B blood-group substances   The enzyme is also present in saliva (Schiff and Weiler, 1931*b*), and is found in the saliva of secretors and non-secretors alike (Matson and Brady, 1936)   The enzyme does not appear to be derived from the bacteria found in the mouth, and it has been suggested by Schiff and Buzón (1935) that the enzyme is secreted by the glandular cells   Landsteiner and Chase (1935) reported that A-substance was decomposed by an organism, *Pullulomyra botrytis* (Thaysen, 1939), which was isolated by Morgan and Thaysen (1933) and shown to be active in decomposing a number of specific bacterial polysaccharides

The isolation of the specific blood-group substances of animal and human origin in a purified form led to a reinvestigation of the enzymic destruction of their serological properties (Morgan, 1946)   A number of *Cl welchii* (Type A) culture filtrates, crude and partially purified with reference to collagenase (κ-toxin), an enzyme which breaks down collagen (Oakley, Warrack and van Heyningen, 1946 , Bidwell and van Heyningen, 1948 , Bidwell 1949) were examined, and were found to contain enzymes which would rapidly inactivate the purified A B and H blood-group substances   Increase in activity of the collagenase hyaluronidase and certain toxins common to these filtrates during their purification and concentration, however was not correlated with increased capacity to inactivate the group mucoids, and it appeared that they were not the factors responsible for the decomposition of the blood-group substances   Nor does the λ-enzyme of Oakley, Warrack and Warren (1948) appear to be involved since after purification of a *Cl welchii* (Type B) culture filtrate with a resultant 50 per cent yield of this enzyme, only 2 per cent of the A-enzyme remained   Heating the culture filtrates for 1 hour at 56° resulted in the inactivation of the enzymes which destroy the A- and B-substances, but left unimpaired the enzyme which decomposed the so-called O-substance, now referred to as H-substance (Morgan and Watkins, 1948)   The results of these investigations indicate that at least two enzymic activities are displayed by the partially purified culture filtrates

The activity which destroys the serological properties of the A and B group substances is thermolabile, the other enzymic activity is relatively thermostable and destroys only the H-substance

The examination of culture filtrates from selected strains of *Cl welchii* (Type B) has shown that enzyme preparations of much greater activity can be obtained readily, and the work described in this communication concerns the purification and properties of the group specific enzyme preparations obtained from Type B strains The intended separation of the enzymic activities has not been achieved, and therefore the A- or B-enzymes cannot yet be obtained free from H-enzymic activity A preliminary account of this work has already appeared (Stack and Morgan, 1948)

EXPERIMENTAL
Materials and Methods

Cultures

The *Cl welchii* (Type B) strain used by Schiff (1939), also strains CN 1253 and CN 1990, were found to yield the most active filtrates encountered Fourteen other strains examined after two subcultures on peptone medium were found to yield not more than one-fifth of the enzyme titre found in the culture filtrates of the three chosen strains Another strain, originally producing less enzyme than these, produced filtrates of maximum activity after three subcultures had been made Culture filtrates from representative Type A, C and D strains contained about one-tenth of the enzyme activity found for average Type B strains Tests on culture filtrates from a few strains of *Cl histolyticum, B anthracis, Staph aureus, Ps pyocyanea* and *V cholerae* failed to reveal the presence of enzymes able to inactivate the blood-group substances

The organisms were grown on a peptone medium consisting of Evans peptone, 3 per cent, and sodium β-glycerophosphate, 2 5 per cent, supplemented before inoculation with glucose, 2 ml per 100 ml of a 10 per cent solution, and thiolacetic acid, 2 ml per 100 ml of a 0 4 per cent solution Without glucose, enzyme yields were generally one-third less, but without thiolacetate, only one quarter of the enzyme production occurred Glucose caused no stimulation of enzyme production unless thiolacetate was present The addition of 0 01–0 20 per cent of the test substrate caused no adaptive response As group substances are present in the peptone used in the medium, the lack of adaptive response was confirmed by growing the same strain in the simplified medium described by Rogers (1945) with or without the addition of purified pig gastric mucin (A- and H-substances)

Specific blood-group substrates

The test-substrate employed, unless otherwise stated, was a purified mucoid material obtained from a commercial preparation of pig gastric mucin according to the method described by Morgan and King (1943) The substance possessed A and H, the so-called O, activity Recent work has shown that the O specificity of the mucoid is not the same as would be expected if the material were a product of the *O* gene For this reason Morgan and Watkins (1948) suggested that the so-called O character would be better designated by the letter H as this symbol would serve to emphasize that it is not a product of the *O* gene, but that it is a

heterogenetic material common to the great majority of human erythrocytes, irrespective of their A B O Group

In a few experiments a preparation of human A-substance was employed which showed no H-activity, and on the basis of its chemical, physical and immunological properties was most probably homogeneous Similarly, carefully purified H-substance obtained from pseudomucinous ovarian cyst fluids was used in other experiments A potent specimen of ovarian cyst fluid obtained from a Group B secretor served as a source of B-substance These materials were readily inactivated by the enzyme preparations

For convenience in recording the results the enzymic activities which destroy the A-, B- and H-substrates are assumed to be due to separate enzymes and are termed A-enzyme, B-enzyme and H-enzyme respectively The properties of the B-enzyme have not been examined in detail, but in those examined they do not appear to be distinguishable from those of the A-enzyme, except in their group specificity

Measurement of enzymic activity

The amount of the specific substrate, present at any given time after mixing with the enzyme preparation, was determined by means of the agglutination inhibition test described earlier (Morgan and van Heyningen, 1944) The anti-A, anti-B and anti-H sera used throughout the investigation were frequently titrated in inhibition tests against " standard " preparations of A-, B- and H-substances, respectively The sera were stored in 2 to 3 ml amounts without antiseptic and in sealed ampoules at − 10°

Ideally, the amount of change taking place over a short interval of time and in the presence of a large excess of unchanged substrate should be measured to determine the activity of an enzyme preparation In this instance, however, measurements of this kind cannot be made owing to the insensitive nature of the agglutination inhibition test, which measures the excess antibody left in the system after the undecomposed group substance has neutralized a part of the standard dose of agglutinin Agglutinin and, therefore, substrate concentrations differing by 50 per cent can readily be determined, and differences in concentration of this order are usually employed in agglutinin titration tests If care is taken and all dilutions are made with graduated pipettes in place of the Pasteur pipettes usually employed in this type of titration, differences of 33 per cent in agglutinin concentration can be detected For the simple purpose of following the change in activity of an enzyme preparation, for example during purification, 50 per cent differences, as derived by making progressively double dilutions of the enzyme-substrate system, are convenient, and have been used in this investigation

A unit of enzymic activity is defined as that amount of activity which will bring about an extent of inactivation of 1 0 ml of 0 10 per cent substrate in 2 hours at 37° and pH 7 0, which results in the lowering of the inhibition titre by four tubes when the usual two-fold geometrical dilution scale is employed This unit is equivalent to the destruction of 94 per cent of the serological activity of 1 0 ml of a 0 10 per cent solution of the blood-group substance

Tests for the measurement of enzymic activity were carried out as follows The group substance, 1 0 ml of a 0 10 per cent solution, was mixed with different amounts of the enzyme preparation contained in 1 0 ml of McIlvaine buffer, pH 6 8–7 0 The mixture was incubated at 37° for 2 hours in the presence of toluene

and then heated for 10 mm at 100°   The latter step is essential, otherwise the enzyme preparation brings about a sensitization and false agglutination of the red cells used in the agglutination-inhibition test subsequently employed to measure the extent of inactivation of the substrate

The results of a typical titration carried out to determine the activity of an enzyme preparation are set out in Table I, from which it will be seen that 0 80 mg of the enzymic material brought about a lowering of the inhibition end-point by four tubes, i e decomposed 94 per cent of the substrate under the standard test conditions   By definition, this amount, 0 80 mg , of the preparation contains one unit of activity   With the same laboratory " standard " a parallel series of results was obtained for the H-enzyme tests

TABLE I —*Showing the Inactivation of A-substance by Enzyme in 2 Hours at 37°, pH 7 0*

Enzyme (mg per ml)	Inhibition of agglutination of A cells by anti A serum after the addition of the following dilutions of 0 10 per cent substrate									Residual substrate (per cent)	Calculated A enzyme (units/mg)
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512		
0	0	0	0	0	0	0	0	1	2	100	—
0 2	0	0	0	0	0	0	1	2	3	50	(1 2)
0 4	0	0	0	0	0	1	2	3	4	25	1 2
0 6	0	0	0	0	1	2	3	4	4	12	1 2
0 8	0	0	0	*1*	2	3	4	4	4	*6*	*1 2*
1 0	0	0	1	2	2	3	4	4	4	3	1 2
1 2	0	1	2	3	4	4	4	4	4	1 6	1 2
1 6	1	2	3	3	4	4	4	4	4	0 8	1 1

0 = Absence of agglutination , 1 = groups of a few cells (the end point) , 2 = larger groups, many free cells , 3 = clumps visible without magnification , 4 = macroscopic agglutination

In view of the insensitive nature of the agglutination inhibition test it is advisable to determine as closely as possible the amount of enzyme which will bring about a lowering by four tubes of the inhibition end-point of the standard substrate, measured under the defined conditions   When many determinations of activity are involved, however, as is inevitable for example in following the efficiency of purification procedures, greater or less inactivation than 94 per cent is frequently recorded, and an empirical interpretation of the end-point titres given in Table I has been used for the determination of the activity of the preparations in terms of the proposed units

It has been found that if the difference in the number (N) of two-fold dilutions required to give the agglutination inhibition end-point before and after the enzyme has acted, be multiplied by the initial quantity of substrate (1 0 mg) and divided by the amount of enzyme used, the result is approximately constant   The above definition of the unit employed can therefore be expressed in the working formula

$$\text{Enzyme units} \frac{N}{4} \times \frac{\text{Initial substrate}}{\text{Initial enzyme}}$$

Table I contains the titration results obtained in one particular experiment in which the enzyme-substrate ratio was varied 5-fold   The calculated activity in units is in each instance the same within the experimental error   Similar

results were obtained when the H-enzyme and its substrate were examined The extent of substrate destruction by an appropriate amount of enzyme was not affected by ten-fold dilution of the system before incubation or by employing an acetate buffer

Enzyme Production during Growth of Cl welchii (Type B)

Culture filtrates show maximum activity as soon as vigorous gas evolution ceases, usually about 12 to 15 hours after inoculation The A-enzyme reaches its maximum titre at this time and then declines, whereas the H-enzyme, which is more stable under these conditions, remains at this optimal level for at least another day The results of numerous experiments showed that the production of the enzyme occurs equally well whether the medium fills small or large vessels The best yields are obtained when a 1 per cent inoculum from a vigorously growing subculture is added to the warm medium, freshly supplemented with glucose and thiolacetic acid Poorer yields of A-enzyme, which, however, showed greater specific activity, could be obtained by growing the organisms in peptone medium contained in a cellophane dialysis sac, surrounded by fresh medium at 37°

Stability of Enzyme Solutions

Cultures were centrifuged and the clear supernatant fluids stored at 0 to 2° under toluene If used within a day or so, the supernatant fluids show good enzymic activity against A-, B- and H-substances If required for use more than a week later storage at − 10° without preservative is essential, but not always successful for the A-enzyme The H-enzyme is fairly stable in aqueous solution at 0° Drying a fresh culture filtrate from the frozen state prevents rapid deterioration of the enzymes Precipitation of the enzymes by the addition of solid ammonium sulphate to the culture medium gives rise to a sticky scum, which partially dissolves in 80 per cent of glycerol to yield a stable enzyme solution The addition of 0 4 per cent of gelatin, gum arabic, or peptone, does not stabilize aqueous solutions of the A- and B-enzymes The stability of the enzymes was examined at pH levels between 3 and 12 by incubating for an hour at 20° before readjusting to pH 7 0 and testing It was found that the A- and H-enzymes retained their full activity under these conditions from pH 5 up to a value as high as pH 11

In confirmation of the earlier results of Morgan (1946), it was found that incubation in the absence of substrate for one hour at 56° destroys the A- and B-enzymes and such heated preparations can be used for control purposes Fig 1 shows that the A- and H-enzymes can be qualitatively differentiated in the one direction by heating at 55° for 10 minutes owing to the relative stability of the H-enzyme in buffer (pH 7 0) at this temperature A preliminary incubation of the enzyme-substrate mixture at 55° or 60° for 10 minutes prior to the usual 2-hour reaction period at 37° reveals that the A-enzyme is protected at 55° by its substrate, but not at 60°, while the H-enzyme is protected at 60°

If, after the usual enzyme-substrate incubation for 2 hours at 37°, a further 1 0 mg amount of substrate be added, it has been found that on repeating the incubation, complete inactivation of the additional H-substance by the H-enzyme occurs However, the A-enzyme is unable to inactivate the extra A-substrate completely—a finding which is not unexpected in view of the more labile character

of the A-enzyme, which presumably loses the protection of its substrate towards the end of the first incubation period

Unit quantities of the purified enzymes in phosphate buffer were incubated for 2 hours at 37° with equal volumes of 0 02 M solutions of the following substances Potassium cyanide, formaldehyde, glycerol, ascorbic acid, phenol, manganous chloride, ferric chloride, lead acetate, and silver nitrate After further incubation with substrate, the H-enzyme titre was found to be enhanced by the first and depressed by the last three substances Inhibition only occurred in tests where precipitation of the metal phosphates occurred The serological test was not affected in blank experiments by the substances which appeared to interfere with the enzyme These results are similar to those reported by Schiff and Weiler (1931*b*) and by Stimpfl (1932)
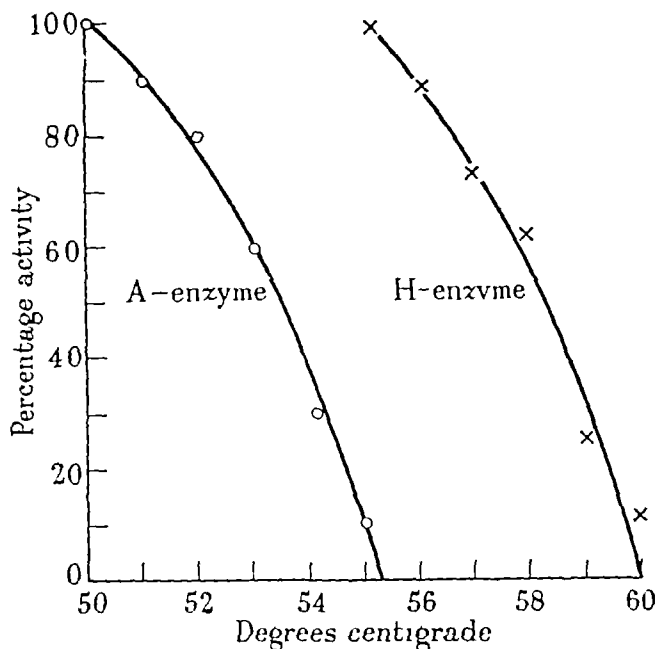
FIG 1 —Differential inactivation of A and H- enzymes by incubation for 10 minutes over the range 50–60°

Optimum Temperature and pH of Reaction

The optimum temperature for the reaction between the A- and H-enzymes and their substrates was investigated by incubation at pH 7 for 2 hours The results indicated that both enzymes are most active over the range 40–45°, and still appreciably inactivate their substrates at temperatures above 50° Fig 2 is a composite curve based on over fifty readings averaged over 5° temperature intervals in order to detect the small differences in activity observed between 30° and 50°, measurements on both A- and H-enzymes being combined, since there were no observable differences between them This conclusion is not at variance with the evidence on temperature stability, for in the latter experiment no substrate was present

The rate of enzyme action at 37° was approximately doubled at 47° and halved at 27° The results set out in Fig 3 show that purified enzymes can be assayed in 10 minutes instead of the 2 hours required for culture filtrates Inactivation

32
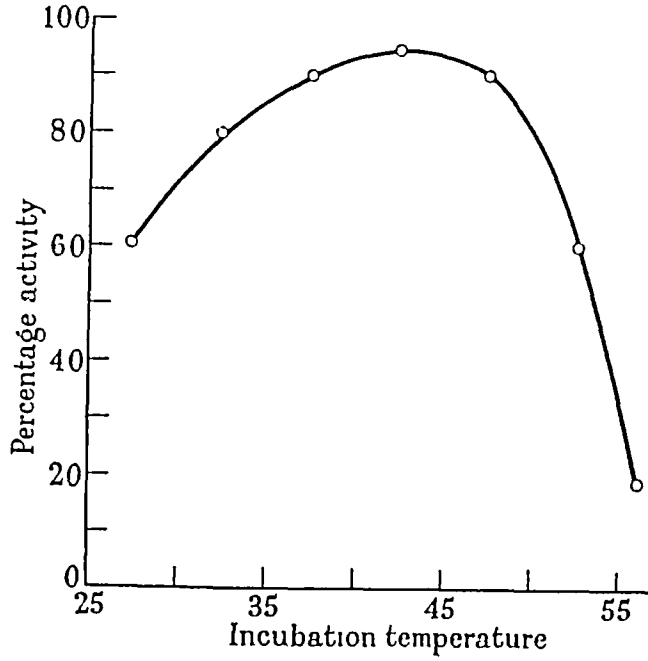
FIG 2 —The variation of enzyme activity with temperature   Showing the mean values over 5° ranges   Extreme values differ by ± 20 per cent from those plotted
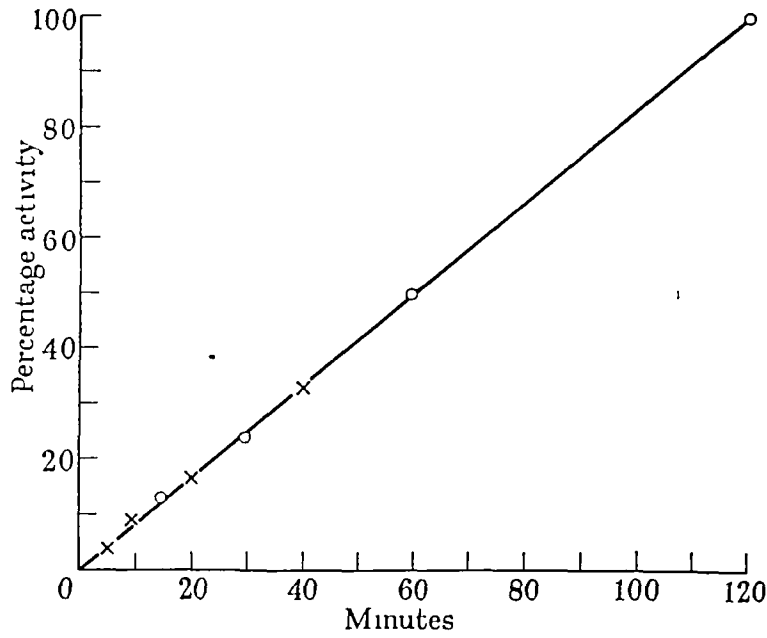
FIG 3 —Showing the relationship between incubation period and enzyme activity calculated from (a) the enzyme substrate ratio, which was progressively halved as the incubation period was doubled, and (b) the constant inactivation of the substrate resulting from this procedure

of the substrate to the same extent was obtained by keeping constant the product of enzyme concentration and incubation period

To determine the influence of pH on the activity of the enzymes a standard enzyme preparation was mixed with 0 10 per cent substrate in the presence of citrate, phosphate and borate buffers to give pH values between 3 7 and 9 8 The mixtures were incubated at 37° for 2 hours, readjusted to pH 7 0 and heated at 100° for 10 minutes before titration for undecomposed blood-group substance The averaged results of a number of experiments compose the curves given in Fig 4, from which it will be seen that there is a steady increase in A-enzyme activity from pH 3 7 to about pH 5 5 The activity then falls until at pH values
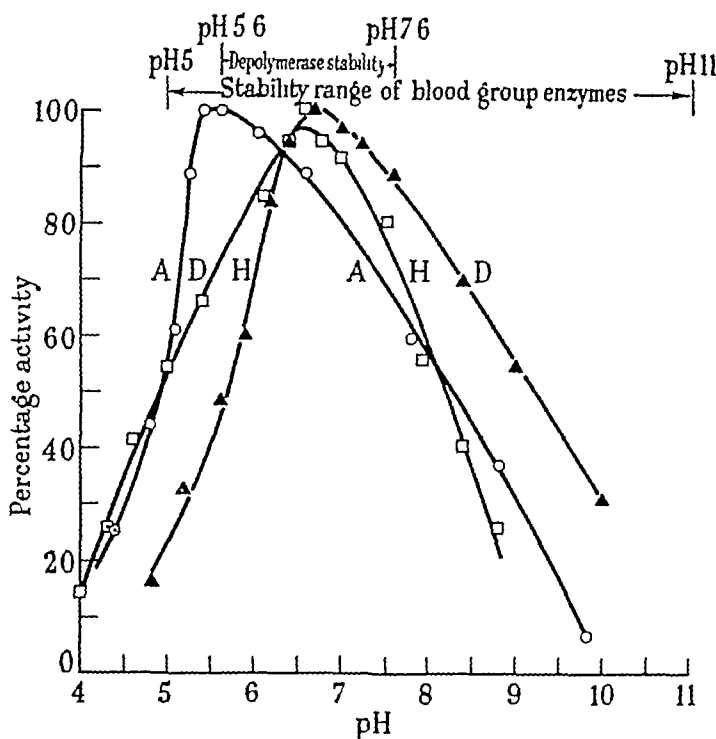
Fig 4 —The variation of enzyme activity between pH 4 and 10   A (O——O) A enzyme   H (▲——▲) H-enzyme ,   D (□——□) depolymerase

above 9 no significant inactivation of the specific substrate occurs   The H-enzyme behaves similarly, but shows its optimum activity at about pH 6 5   In both instances, however, over 90 per cent of the maximum activity is shown over a range of 1 2 pH units   In following the isolation and purification of the enzymes which will now be described, the measurement of enzymic activity has been carried out within the pH range 6 8–7 0, which provided a favourable condition for enzyme action, destruction of residual enzyme, and titration of residual substrate

The Purification of the Blood-Group Enzymes

The assessment of the specific activity of materials obtained as a result of fractionation of crude culture supernatant fluids was made by expressing the activity in terms of mg " tyrosine ' or mg of nitrogen   Tyrosine ' was deter-

mined by means of a photo-electric colorimeter after the addition of sodium carbonate and Folin and Ciocalteu's reagent (1927) to samples containing 20–40 μg " tyrosine " (light filter Ilford 204) Nitrogen was similarly determined with Nessler's reagent (light filter Ilford 302) after samples of the enzyme preparations, containing 20–40 μg N, had been digested with H_2SO_4 and H_2O_2, using a procedure similar to that described by Miller and Miller (1948) The more convenient estimation of " tyrosine " was permissible, since the quantity of nitrogen present in purified material was consistently 3 7–3 8 times greater than the " tyrosine " content

The addition of 70 g per cent $(NH_4)_2SO_4$ to fresh culture supernatant fluids gives rise to protein scums which are partly soluble in aqueous buffer at pH 7 By this means the specific activity is increased about eight-fold for a 30 per cent recovery of the enzymes

Further similar experiments demonstrate that the most active material, 50 H-enzyme units per mg tyrosine, is thrown out of solution between 40 and 43 per cent added $(NH_4)_2SO_4$, but this fraction accounts for only a small part of the total activity

Attempts to remove impurities from crude enzyme preparations by simple dialysis were without success owing to the extensive loss of enzymic activity Ultrafiltration by means of dialysis sacs, strengthened by silk jackets and kept under reduced external pressure, reduced the fluid to one-tenth of its original volume A considerable degree of purification is achieved by this means, but again the total activity recovered indicates that an extensive loss occurs

Shaking culture filtrates or partially purified material in McIlvaine buffer at pH 7 with alumina, bentonite, calcium phosphate, Celite, Decalso, Fuller's earth, kaolin and magnesium silicate failed to bring about a separation of the enzymes or to remove useful amounts of impurities Passage of the mixed enzymes through columns of charcoal-glass powder mixtures usually resulted in the adsorption of at least 50 per cent of the A-enzyme activity, together with 30 per cent or so of the total solids in the preparation Passage through columns of alumina or calcium phosphate, however, allowed half the H-enzyme to be recovered in the filtrate with very little A-enzyme On no occasion was there evidence that the adsorbed enzymes could be eluted by altering the pH or by running moderate concentrations of electrolytes through the columns

Attempts to adsorb the enzymes on charcoal in the presence of 3M $(NH_4)_2SO_4$ were more successful, for subsequent elution from the charcoal with 1M $(NH_4)_2SO_4$ or buffer at pH 7 0 gave satisfactory yields of enzymes of considerably enhanced purity For example, 1 0 g of dried crude culture filtrate was dissolved in 20 ml 3M $(NH_4)_2SO_4$ and shaken gently with 0 40 g charcoal The charcoal was recovered and eluted successively with 2M $(NH_4)_2SO_4$, 1M $(NH_4)_2SO_4$ and water As 1M $(NH_4)_2SO_4$ appeared to have only a slight influence on the agglutination inhibition test, the two $(NH_4)_2SO_4$ eluates were reduced below this salt concentration by brief dialysis at 0–3° The behaviour of the enzyme dialysed after exposure to similar concentrations of $(NH_4)_2SO_4$ in the absence of charcoal served as a control There is an increase in the specific activity of the control material as a direct result of simple dialysis The results show that when crude enzyme preparations which possess one H-unit associated with one mg " tyrosine " are adsorbed in the presence of 3M $(NH_4)_2SO_4$ and eluted by 1M $(NH_4)_2SO_4$, half of the enzyme is recovered in the eluate and the material so obtained then shows

about 40 H-units per mg " tyrosine " Table II indicates the degree of duplication obtained in consecutive experiments, the pairs of figures for specific activities showing good agreement

TABLE II —*Showing the Activity of Enzyme Preparations Eluted from Charcoal by Reducing the Ammonium Sulphate Concentration*

Fraction tested	Ammonium sulphate (M)	H units per cent	H-units/mg tyrosine
Original	0	(100)	1 , 1
Control	3	60 , 70	3 , 3
Eluate A	2	6 , 3	5 , 4
Eluate B	1	38 , 23	40 , 40
Eluate C	0	6 , 4	7 , 8
Filtrate	3	10 , 15	1 , 1
SUM	—	60 , 45	3 , 3

The addition of 3 volumes of acetone to the crude filtrates as suggested by Schiff (1939), who employed a different medium, resulted in the deposition of an oily layer without giving rise to a significant increase in the specific activity of the material thrown out of solution   Treatment of fresh culture filtrates with an equal volume of acetone at 0° gave a satisfactory increase in purity with a yield of 70 to 80 per cent of the original activity   Methanol and ethanol have been employed with similar results   This method was used to prepare a quantity of the mixed enzymes suitable as a laboratory " standard "   The details for preparing a useful quantity of enzymic material by this process are as follows

Conical flasks filled nearly to the neck with 950 ml of sterile peptone medium were inoculated and incubated overnight at 37°   " Superaid," 0 5 per cent, was added and the culture fluid filtered through paper   After cooling in an ice-salt bath the filtrate was mixed with an equal volume of acetone which had been cooled to − 30°   Regulation of the rate of acetone addition during the 10–15 min required to complete the process ensured that the precipitation took place at 0°   Material thrown out of solution was collected by centrifugation and extracted with 25 ml of cold McIlvaine buffer at pH 7 0   In six such experiments the average yield of the enzymes, purified some 150 times with respect to nitrogen or " tyrosine," per unit of activity, was not less than 60 per cent   Further extraction of the residue with buffer after the first treatment yielded additional material of lower specific activity   The main extracts were immediately shell-frozen in large round flasks and dried at 0 1 mm pressure from the frozen state

TABLE III —*Showing the Activity of Acetone-Precipitated Blood Group Enzymes*

Original (As A and H units/g)	A enzyme (units/g)	H enzyme (units/g)	Depolymerase* (units/g)	Nitrogen (mg /g)
25	26†	24†	11†	70
25	1700	1200	450	31
36	1600	1600	640	30
48	2600	2400	440	22
58	2900	2900	500	18

* After storage for 3 months
† Crude filtrate not acetone precipitated

Thorough drying *in vacuo* over P_2O_5 of the white powders so obtained was followed by transfer to Macartney bottles at 0°   It was noticed, however, that not more than half the initial activity remained after two months' storage under these conditions   Details of the acetone precipitation of 4 culture filtrates are set out in Table III   The corresponding figures for a crude filtrate are also included   A carefully purified preparation contains at least 120 units of A- and H-activity per mg nitrogen   Reprecipitation of material showing this order of activity with 0 4–1 3 volumes of acetone under the above conditions did not significantly increase the purity

Immunization of Rabbits with Enzyme Preparations

As a result of the failure to obtain preparations of A-enzyme devoid of power to inactivate H-substance by the methods described, an attempt was made to immunize rabbits with H-enzyme with the hope that an anti-H-enzyme would be produced which could be used to block the specific activity of H-enzyme without influencing the A-activity of the preparation   To this end rabbits were immunized with the unheated enzyme and with preparations heated at 56° for 10 min , which showed considerable specific A- and H-, and H-enzymic activity respectively

The toxic nature of the material injected caused the death of four of the experimental animals, but one animal in each of the two groups survived the immunization and received a total of 1 6 mg of enzyme protein over a period of 4 weeks   The natural A- and O-agglutinins were removed from the rabbit sera and suitable amounts of the sera were included in the standard test for enzyme activity   The results are given in Table IV, and show that on mixing the rabbit serum with the enzyme before the addition of substrate, some of the enzyme was neutralized or inactivated, for less enzymic activity was found than was added Normal serum had no such effect

TABLE IV —*Showing the Neutralization of Enzyme by the Serum of Rabbits Immunized with* (a) *Heated and* (b) *Unheated Enzyme Preparations*

Antigen (enzyme)		Antiserum used (ml)	Units present	Units found
Heated	{	0 50	1 0A	0
		0 50	1 5A	0 5
		0 25	1 5A	1 0
		0 50	1 7H	0
Unheated	{	0 50	1 5A	0
		0 25	1 5A	0 S
		0 12	1 5A	1 2
		0 50	1 7H	0

The Enzymic Depolymerization of Pig Gastric Mucoid

Specimens of mucoid isolated from commercial hog gastric mucin frequently show a high viscosity, and it has been observed that during the enzymic inactivation of the A and H serological properties of this material, a rapid fall in viscosity occurs   In our experience specimens of A- or H-mucoid isolated from individual pig stomach linings by peptic autolysis, and the A- and H-substances recovered from human ovarian cyst fluids, show no significant viscosity   Artificial

mixtures of A- and H-substances which individually show low viscosity are also non-viscous It would seem, therefore, that a component of the commercial mucoid which is neither A- nor H-substance is responsible for the high viscosity of this material It is of interest that the " depolymerase " present shows many properties which are similar to those of the group enzymes, and has been found to accompany these enzymes to the same extent after they have been purified as much as two hundred-fold in terms of the A- and H-enzyme activities (Table III) The highly viscous substrate is not depolymerized by hyaluronidase, and solutions of hyaluronic acid are not rendered less viscous by the blood-group enzyme preparations Unlike hyaluronidase, the *Cl welchii* depolymerase does not require optimum salt and buffer concentrations The behaviour of buffered solutions of the depolymerase in the presence of the mucoid was followed in Ostwald pattern viscosimeters over a period of about 30 minutes at 37° During this time a typical *Cl welchii* (Type B) filtrate will reduce the relative viscosity of an equal volume of 0 25 per cent gastric mucoid from 1 6 to 1 3 The amounts of enzyme and substrate chosen were governed by the fact that at substrate concentrations greater than 0 25 per cent, the relationship between flow time and substrate concentration in the absence of enzyme ceased to be linear The quantity of depolymerase present in the *Cl welchii* culture filtrates decreased steadily after reaching a maximum at the end of 24 hours' growth The pH-activity curve for this enzyme and the pH-stability range, which is considerably narrower than that of the blood-group enzymes, are shown in Fig 4 Behaviour similar to that of the blood-group enzymes was noted on adsorption and precipitation, e g one-third of the depolymerase was recovered from culture filtrates by adding 70 per cent ammonium sulphate, the specific activity being increased 8 times, whereas the acetone purification procedure described above improved the specific activity some two hundred times Hydrogen peroxide, potassium cyanide, and ascorbic acid, 0 02 M, caused some inhibition of activity Toluene and merthiolate could safely be used as preservatives for the enzyme preparations, which were found to be stable for several weeks in the refrigerator

DISCUSSION

The action of the enzymes present in the culture supernatant fluids of certain strains of *Cl welchii* (Type B) has been examined, using preparations of blood-group substances of animal and human origin as substrates

The enzyme activities can be differentiated by heating the mixed enzyme preparations for 10 minutes at 55°, whereby the power to destroy the A and B serological character is lost The capacity of the resulting solution to inactivate the H-substance is, however, unimpaired, and quite potent preparations of H-enzyme free from A- and B- activity can be obtained in this manner Attempts to separate the enzymic activities by fractional precipitation techniques that involve the addition of ammonium sulphate, organic solvents or certain adsorbents to the crude culture filtrates have been, from a practical point of view, largely unsuccessful Similarly, adsorption on charcoal columns failed to bring about a useful separation of the enzymes By applying certain of these procedures, however, the specific activities related to total nitrogen or " tyrosine " were considerably increased Nevertheless, no useful separation of the enzymes was attained either from each other or from the depolymerase

The properties of the enzymes most thoroughly studied are summarized in

Table V   It might be deduced from the results given there that the depoly-merase could be freed from blood-group enzyme activity by dialysis, and that the depolymerase could be removed from the blood-group enzymes by allowing the preparations to stand at pH 11 for an hour at room temperature, but we have made no attempt to accomplish these changes

TABLE V —*Summarizing the Properties of the Blood Group Enzymes and Hog Mucin Depolymerase*

Property	A enzyme	H enzyme	Depolymerase
pH optimum	5 5	6 5	6 8
Half activity, pH	4 9, 8 2	4 9, 8 3	5 6, 9 1
pH stability range	5–11	5–11	5 6–7 6
Temperature optimum	40–45°	40–45°	38°
Inactivation temperature (10 min)	55°	60°	56°
Half activity, temperatures	25° 53°	25° 53°	33° 50°
Growth optimum (days)	1	1	1
Stability on dialysis	±	±	+
Stability on storage (liquid)	−	±	±
Average purification ratio (acetone)	200	200	200

The general lack of distinction between the physical properties of the blood-group enzymes is of interest   It could be surmised that certain groupings on the enzymic complex which are specific for the A- or B-substrate or responsible for the depolymerizing activity become denatured, leaving other groups unaffected and still able to bring about the inactivation of the H serological character   The evidence in support of such a conclusion is, however, far from complete   The inactivation by the enzyme preparation of the specific mucoids associated with the " Lewis " blood-group characters, Le^a and Le^b (Mourant, 1946 ,   Andresen, 1947), has already been recorded (Grubb and Morgan, 1949)

The purified A-enzyme destroys the specificity of Group A mucoid when this character is measured by the haemolytic inhibition test, and in this property it differs from that shown by some crude enzyme preparations obtained from other types of *Cl welchii* (Morgan, 1946) which failed to accomplish the destruction of A-specificity when measured by this technique   It is to be noted, however, that the differentiation of the two serological properties, the power to (*a*) inhibit iso-agglutination and (*b*) prevent the haemolysis of sheep-cells by rabbit serum, can be brought about by other means   Thus, it has been shown that after treat-ment with dilute acid the former activity is destroyed, whereas the latter activity is enhanced (Aminoff, Morgan and Watkins, 1948)

A strictly quantitative interpretation of the enzymic inactivation of the group substances in terms of the inhibition of agglutination is not straightforward when so little substrate remains, and when the indicator systems are relatively insensitive to changes in concentration of the reactants   The formula used to determine the units of activity when smaller or greater amounts than 94 per cent of the substrates are de-composed is based on the assumption that the reaction is not seriously affected by the almost complete destruction of substrate, and although primarily empirical, is of considerable practical value, and enables one to avoid making several titrations to determine the amount of the enzyme preparations required to bring about a lowering of the inhibition end-point by exactly four tubes

Information about the chemical changes induced in the group active mucoids by the enzymes cannot readily be obtained when excessive amounts of crude enzyme material must be employed to destroy the serological character of these substances The purified enzymes now described contain one-tenth as much nitrogen per unit as 1 0 mg of the specific blood-group mucoid They are, therefore, suitable for investigations of this kind and are being employed for this purpose Similarly the action of the enzymes on the group specific character of stroma and intact erythrocytes is being studied

SUMMARY

The production from cultures of *Cl welchii* of enzymes which destroy the specific serological characters of the human blood-group substances is described

A quantitative method for measuring the activity of the enzymes has been applied to a study of their properties The optimum pH values for the A- and H-enzyme activities are 5 5 and 6 5 respectively, and both enzymes are stable over the pH range 5–11

A considerable purification of the enzymes was accomplished by the procedures described, and preparations containing 60 per cent of the original activity associated with 0 2 per cent of the crude filtrate nitrogen were obtained These enzyme preparations, however, are not always stable when stored in the liquid or freeze-dried condition

The serum of rabbits immunized with the purified enzymes developed weak anti-enzyme properties

An additional enzyme which accompanied the group enzymes depolymerized an unidentified substrate in the "standard" preparation of hog gastric mucin The enzyme is stable between pH 5 6 and 7 6, and shows an optimum activity at pH 6 8

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THE SEROLOGICAL ANALYSIS OF MIXED FLOCCULATING SYSTEMS BY MEANS OF DIFFUSION GRADIENTS *

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SEROLOGICAL analysis differs from other analytical procedures in that by itself it can demonstrate no more than a lack of homogeneity   The method to be described here combines specific flocculation with diffusion of antigen and antibody through a matrix so as to reveal the presence of individual systems within a mixture   The resulting flocculation can be identified by interactions with known flocculating systems

The Limitations of Serological Characterization

Detection of impurities

The specificity of serological reactions needs no emphasis   Substances, such as the egg albumins of birds so closely related as the hen, turkey and guinea-fowl, which are beyond differentiation even by their electrophoretic mobilities, may yet be distinguished serologically   The weakness of serology is that in practice it is based on trial and error   a substance pure by chemical criteria is used to make an antiserum, which is then used to detect the homologous substance in solution Nevertheless, because of serological sensitivity and specificity, "impurities" can in this way often be detected in preparations which have passed all other tests There must be some preliminary idea, however, of the substances to be sought, for though the character of impurities may often be suspected from the nature of the original material and the methods of purification used, frequently the final and purest material obtained by physico-chemical means is a mixture which offers no hint whatever of its constituents

Cross reactions

There is another serological test for homogeneity   If one antigen reacts with serum prepared against a second antigen, both are regarded either as being identical or as containing an identical component, in the latter case the antigen being a mixture   If the first antigen is identical with the second it will, suitably mixed, remove all the antibody from the serum   For obvious reasons the reversed experiment will hold, and a serum prepared against the first antigen should also behave similarly   The weakness of this approach lies in the fact that if both antigens are identical mixtures they may behave as though they were homogeneous

* The subject matter of this paper formed part of a thesis submitted to the University of London in July, 1948, for the Ph D degree

Irregularities in quantitative tests

Multiple zones of flocculation by the tube method are accepted as evidence of a mixed system, but usually little can be learnt about the number of antigens present in the mixture, since closely spaced individual zones coalesce into a single broader zone   Furthermore, many of the observations on flocculative behaviour have been made with systems now known to be impure, and deductions so based may well be erroneous   It is doubtful whether a substance pure in the serological sense has so far been employed in the study of flocculative behaviour, for evidence of purity has always been chemical or serological and subject to the limitations outlined above   The purest substance, from the serological standpoint, which has been used for flocculation experiments is diphtheria toxoid   This is well known to show a rather odd flocculative behaviour and is regarded as an exception , it may well be that its behaviour is the rule and not an exception, the " rule " having been deduced from observations on mixtures

Errors Inherent in the Tube Technique of Precipitation

It is customary to carry out flocculation reactions with the volumes constant Variation of the relative proportions of antigen and antibody is achieved by using a unit volume of one reagent and adding varying dilutions of the other The fundamental observation concerning flocculation by the tube technique was that the velocity of the reaction was greatest at fixed proportions of the two reagents   That this indeed was of fundamental importance was further borne out by the fact that the optimal proportions by flocculation showed close agreement with the neutralization of toxins and with the complete removal of antigens showing no indicator effect   Closer investigation of various systems led to the unexpected fact that in some cases constant antibody (alpha) and constant antigen (beta) titrations showed different optimal proportions   This has been regarded as of theoretical importance, but as most of the observations were carried out in tubes with fixed volumes, the essentially artificial nature of the approach was not appreciated

The limitations of the method become clearer if a hypothetical beta titration is examined (Fig 1)   In this it is assumed that one unit of antigen is equivalent to one unit of antibody, and therefore this combination has the greatest flocculation velocity   The steps of the antibody dilution are successively 1 5 times greater   Instead of the dilution factors, however, arbitrary antigen and antibody units have been used   It is clear that the 7th tube contains the two reagents in equivalent quantities   This is the tube therefore which will have the greatest reaction velocity (since the equivalence was chosen on this basis)   Tubes on either side will show a lesser degree of precipitation representing reduced reaction velocity, until on either side a point is reached where the velocity is—for practical purposes—nil   Thus Tubes 1, 2 and 3 show no precipitation, and this is known as the zone of antibody excess   Similarly Tubes 13 and 14 are clear   This is the zone of antigen excess   The zone of precipitation is seen to be assymmetrical If Tube 5 is now examined, a moderate degree of flocculation is found   At the time of mixing it contained 100 antigen and 200 antibody units a ratio of 1 2 Assuming each unit of antigen to unite with one unit of antibody—a fact which seems to be warranted from the behaviour of the optimum Tube 7—by the time 99 units of antigen have united with 99 units of antibody the ratio would be

deteriorated to 1 101 A glance at Tube 3 shows that a ratio of 1 4 no longer flocculates, and consequently the reaction would have stopped—or taken a different course—long before the antigen excess of 1 101 was reached Similar considerations apply to the antigen excess part of the precipitation zone In Tube 11 the initial ratio of 4 parts of antigen to 1 part of antibody is compatible with flocculation, and may be thought of as gradually altering until theoretically the ratio of 76 units of antigen to 1 unit of antibody is reached Tube 13 shows, however, that 8 units of antigen and 1 unit of antibody will no longer flocculate, and the reaction must stop long before attaining a ratio of 76 1

In fact the composition of the precipitate varies with the proportions of the reagents, the reaction taking a different course in each tube Each of these different reactions must have a reaction velocity smaller than that of the combination in the optimum tube, since the equivalence was arbitrarily chosen at

1	2	3	4	5	6	7	8	9	10	11	12	13	14	
100	100	100	100	100	100	100	100	100	100	100	100	100	100	A
800	600	400	300	200	150	100	75	50	37 5	25	18 75	12 5	6 25	G
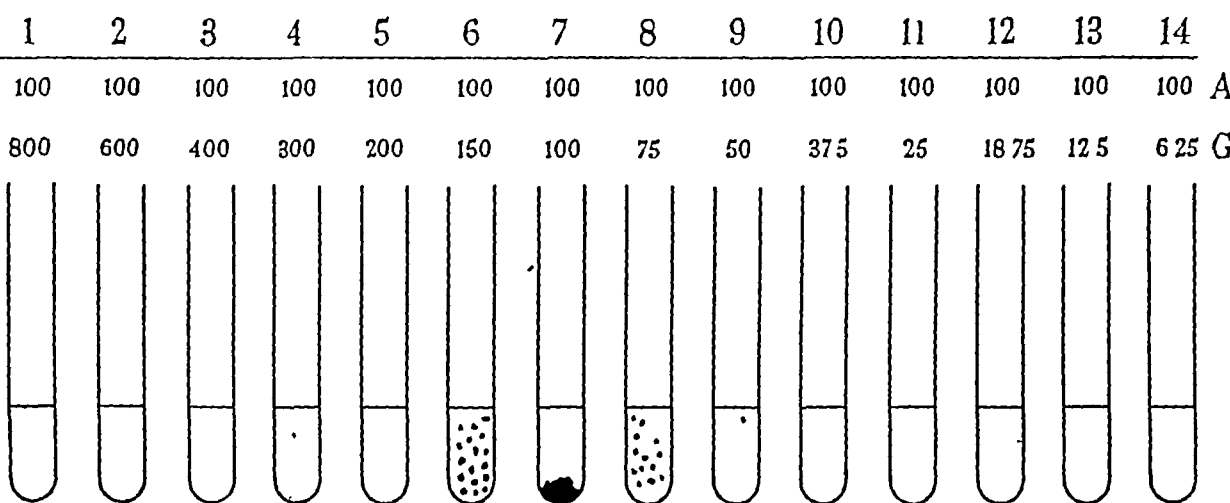
FIG 1 —Constant antigen titration of a hypothetical flocculating system The figures refer to units per unit volume, and it is assumed that one unit of antigen is equivalent to one unit of antibody

the greatest velocity It might be argued that the solubility equilibria of the compounds formed in the various tubes determine the result, but this view is not essentially different from the dynamic concept, and has the disadvantage of expressing results in terms that are not directly obtained from the experiment This is the point of weakness of the tube method that in a series of tubes an essentially different reaction takes place in each In every tube, except the optimum, the system is under a distortion, which tends to become more accentuated as the reaction progresses The true optimal ratio is not likely to be included in any set of titrations using a finite number of tubes, but will usually lie either to the right or to the left of the "optimal" tube, and the distorting effect described will come into play These considerations already constitute a criticism of the tube method If it is now assumed that the systems are multiple, involving several pairs of antigen and antibody, then there are several principal velocities, each surrounded by its own family of secondary velocities The term "secondary velocities" is used to denote the reaction velocities occurring at other than optimal proportions It is here suggested that the principal velocity

—the reaction velocity of the combination at optimal proportions of a given pair of reagents—is the fundamental feature of precipitin reactions This may, however, be obscured in the case of multiple systems with optima so close to each other that a single zone is formed The apparent optimum in such a case is misleading

To sum up, the tube method is ill suited to the accurate determination of the velocity optimum of even a single theoretically pure system, and the characteristic velocity curves of multiple systems may be completely buried in what appears to be a single wide zone However, it is intended to show how a system may be set up in which the particles of each reagent are free to choose their partners at any concentration, and the optimal proportions are obtained under dynamic conditions

The Combination of Flocculation with Diffusion

The law of optimal proportions manifesting itself in the form of a zone in the tube method formed an obstacle to the use of flocculation for the qualitative demonstration of either antigen or antibody The earliest attempt to overcome the zone effect was the introduction of the ring test When a serum is overlayered with a dilute antigen, the resulting precipitation is largely independent of the relative proportions of the reagents The principle of this test is that an interface is formed between the antigen and antibody solutions, and from this plane antibody will diffuse upwards and antigen downwards In this way concentration gradients will be set up, which, with the mixing involved in the process of over-layering, will produce conditions at some level including proportions capable of flocculation This simple technique held the pride of place in precipitin reactions for the first quarter of this century, not because its principle was understood, but on account of its ready performance, and its applicability to the detection of minute amounts of antigen Its circumvention of the zone phenomenon became obvious only in retrospect when the importance of the relative proportions in flocculation reactions had been recognized

A gel as a matrix for combining diffusion with flocculation appears to have been first used by Bechhold in 1905 He incorporated gelatin into an antiserum, and after setting overlayered it with the antigen which was goat serum After a time two sharply separated rings appeared which he believed to mean that the Liesegang phenomenon had been reproduced with colloids Similar observations were reported by Reiner and Kopp (1927) The multiplicity of antigenic components which would afford an alternative explanation of such findings appears to have been completely neglected in the study of precipitin reactions until recently Nicolle, Cesari and Debains (1920) and Hanks (1935) mixed antigen with gelatin and overlayered it with serial dilutions of the antibody Petrie (1932) combined nutrient medium with diffusion matrix An antiserum was incorporated into nutrient agar on which colonies of homologous organisms showed surrounding rings of opacity, and streak inocula showed lines on either side These opacities were thought to be due to flocculation between specific polysaccharide, liberated by autolysis and antibody in the agar base itself The nature of the reaction was confirmed by model experiments in which homologous purified polysaccharide was dropped on an agar base containing pneumococcal antiserum Sia and Chung (1932) working independently on the in vitro transformation of types used the same method Petrie and Steabben (1943)

applied the method for the recognition of toxicogenic clostridia by using a medium specially suited for toxin production and adding antitoxin to it Concentric rings were accepted as a positive reaction, and were believed to be due to the Liesegang phenomenon Multiplicity of the toxins was not suggested as an explanation They were less successful with *C diphtheriae*, but Ouchterlony (1948) reported reasonably good agreement between this method and the guinea-pig test In model experiments he showed that streak inocula across a ditch containing antitoxin yield lines which show joining Elek (1948) obtained an antitoxin gradient by a filterstrip moistened with antitoxin and sunk into a medium specially designed for toxin production It was found that this method
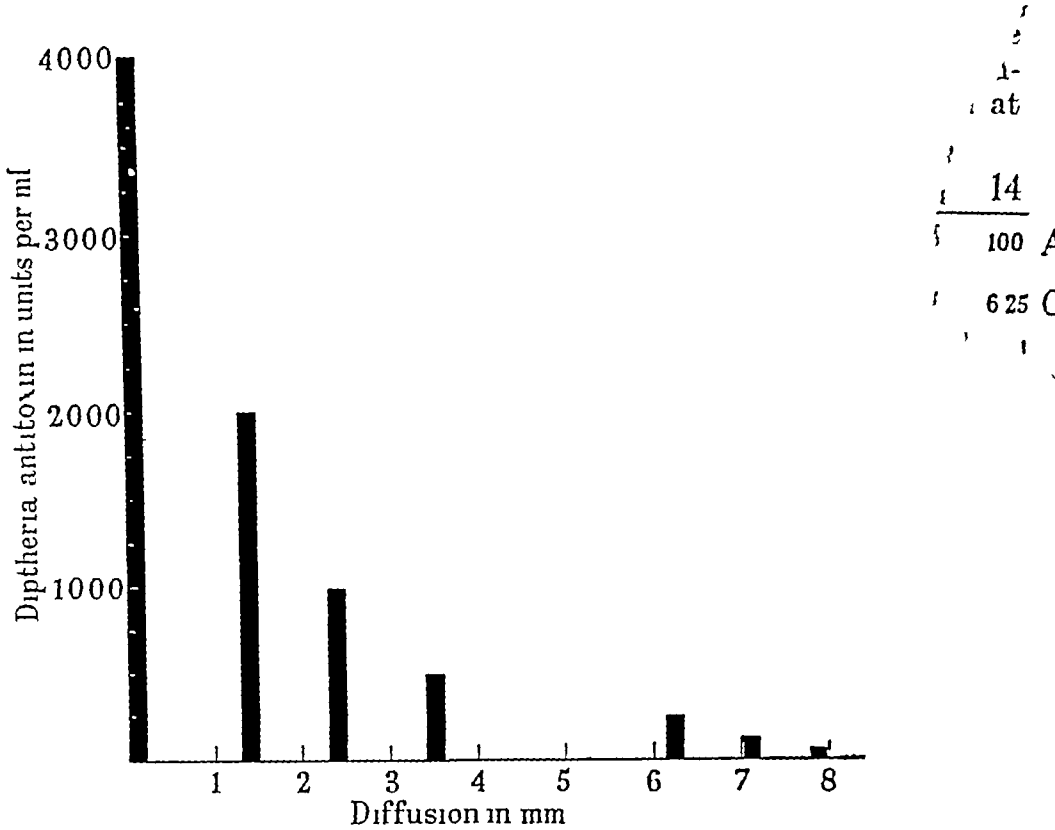
FIG 2 —Diffusion gradient of diphtheria antitoxin in agar after 48 hours' incubation at 37° C

was applicable to *C diphtheriae* and to staphylococci The latter gave multiple lines suggesting a multiplicity of antigens This system was free from non-specific opacities which may occur on serum media Carter and Wilson (1949) confirmed the practical usefulness of this test , Ouchterlony (1949) in a later paper also reported good results with a larger series which included ditch plates

Thus diffusion can be utilized in two ways One reagent can be incorporated into a matrix, and the other may be allowed to diffuse producing a concentration gradient A system of this sort will be free from the zone effect as long as the concentration gradient includes the optimal proportions for the fixed concentration in the matrix This, however, involves the preparation of several plates with various antitoxin concentrations, as in the methods of Petrie and Steabben (1943) and Ouchterlony (1948) The other way is to allow diffusion of both

reagents so that two concentration gradients are formed in the matrix   This may be referred to as the double-diffusion-gradient method, and has considerable advantages over the first in that it produces conditions not only for the interaction of antigen and antibody in all proportions, but also for the maintenance of a continuous and progressive reaction

A concentration gradient of a diffusible reagent is produced by incorporating it at one point in the shallow layer of agar in a Petri dish   If a filterstrip soaked with a concentrated antibody or antigen is used, the concentration gradient due to diffusion can be visualized in the form of orbits, points of equal concentration being equidistant from the edge of the strip   In actual fact only a small part

Diptheria antitoxin 1000 units per ml
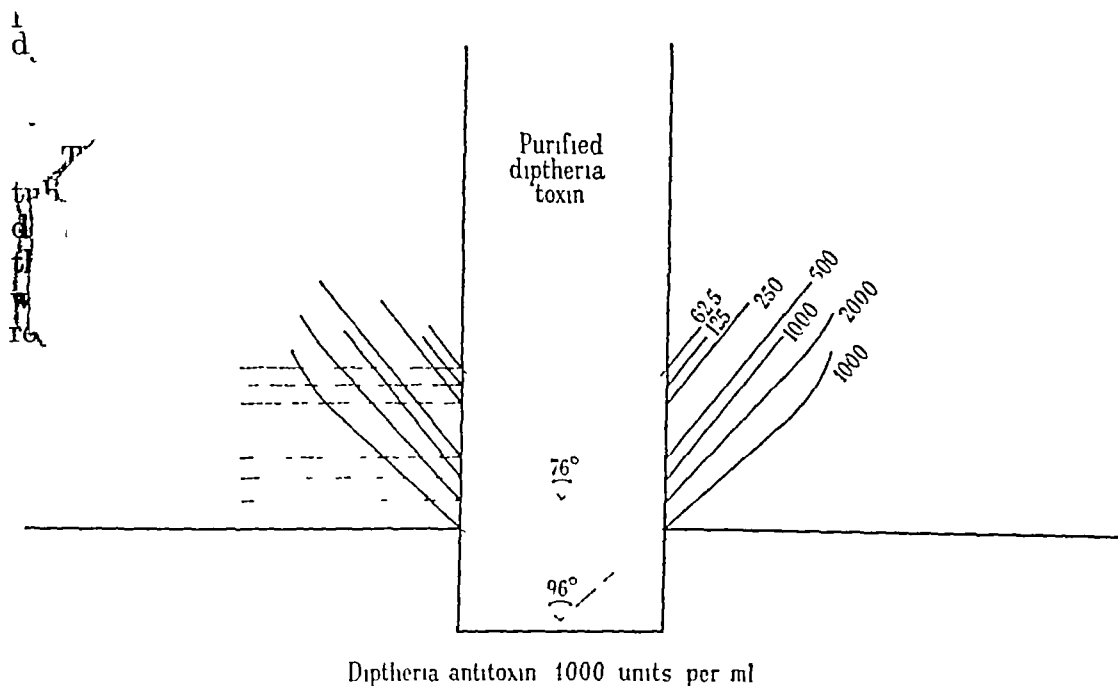
FIG 4 —Composite diagram showing the effect of varying the ratio of antigen to antibody   The antitoxin was kept constant at 0 1 ml of 4000 units per ml   The toxoid concentration was varied from 4000 units per ml to 62 5 units per ml , 0 1 ml of each dilution being used

of these orbits can exist on account of the shallow layer of the agar   The sections of the orbits will be at right angles to the surface, and for practical purposes lines parallel to the edge of the filterstrip represent equal concentrations   The only exception is the region next to the filterstrip   Here for a few millimetres conditions are different, as the position in depth of the strip influences the angle of the orbit and its face will not be at right angles to the surface   The effect of this tilting will be referred to later

After the setting of the agar a similar strip soaked with 0·1 ml of purified toxoid of 4000 units per ml was placed on the surface of the first plate at right angles to the other strip   Doubling dilutions of the toxoid were prepared, and a series of plates made by varying the concentration of the toxoid and keeping the anti-toxin concentration steady at 4000 units per ml   Fig 3a shows the result with 0 1 ml of 500 units per ml of toxoid, and Fig 3b with 0 1 ml of 4000 units per ml   It will be noted that the points of insertion of the lines are altered by different concentrations of the reagents   This method furnishes a convenient means of determining the concentration gradients in the plate   After 48 hours' incubation the plates were placed on photographic paper and contact prints were made The lines thus obtained were superimposed and the diagram shown in Fig 4 was obtained   The points of insertion of the lines and also the angles formed can be seen to vary   The variation is from 96° with 4000 units of toxoid per ml to 76° with 62 5 units per ml   The point of insertion of any line into the toxoid strip represents the equivalent concentration of antitoxin at that point, assuming that over the area of the toxoid trip itself the concentration is uniform—a valid assumption for practical purposes   The distances of the points of insertion from the antitoxin strip can be measured and the antitoxin gradient thus plotted Strictly speaking, as the lines are not formed quite simultaneously, the "observed" gradient will represent a slight distortion from the true gradient, but the actual difference in the values obtained is likely to be within the experimental error of the method   If lines parallel to the edge of the antitoxin strip be drawn from the points of insertion of the various flocculation lines in Fig 4, these will represent antitoxin concentrations corresponding to the flocculation lines from which they originate   These lines will cut the other flocculation lines, and the points of crossing will yield the toxoid concentration at that point   In this way values

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EXPLANATION OF PLATES

Fig 3a —Line of flocculation produced by the double diffusion gradient method   Vertical strip contained 0 1 ml of purified diphtheria toxoid, strength 500 units per ml   The hori-zontal strip contained 0 1 ml of diphtheria antitoxin, strength 4000 units per ml   Incubation 48 hours at 37° C

Fig 3b —Experiment as 3a, except that the vertical strip contained 0 1 ml of toxoid, strength 4000 units per ml   Note that the line of flocculation is nearer to the horizontal filterstrip

Fig 9 —Flocculation of diphtheria toxin antitoxin and toxoid antitoxin systems demonstrating looping

Fig 10 —Plate showing various diffusible antigens by two different strains of *Staph aureus* Flocculation lines due to the same antigen show looping   One of the lines produced by " W 46 " can be seen cutting right across the line of growth of " CN 338," indicating that the latter does not produce this antigen

Fig 11a —Plate showing lines produced by *Cl welchii* type A with polyvalent anti gas gangrene serum

Fig 11b —Similar experiment to Fig 11a, but incorporating human serum   The Nagler effect is clearly shown, and is demarcated by the flocculation lines furthest from the filterstrip

Fig 12 —Agar plate incorporating sheep red cells showing a strain of *Staph aureus* producing two haemolysins   One shows marked inhibition and the other very little

Fig 13 —Agar plate incorporating laked horse red cells inoculated with four strains of staphylo-cocci   Strain " 6 " shows a turbidity which appears to be specifically inhibited by the anti toxin

Fig 14 —Antibody gradient produced by " feeding " the free edge of the filterstrip   The anti-human serum reveals five serological fractions in the human serum used as antigen

Fig 15 —Successive amounts of anti human serum were dried into the horizontal filterstrips The vertical filterstrips contained the following   No 1 = " albumin " fraction of human serum , No 2 = complete human serum , No 3 = " globulin " fraction   This anti-human serum appears to be mainly anti-globulin

3a

3b

9

10

11a   11b

12

13

15
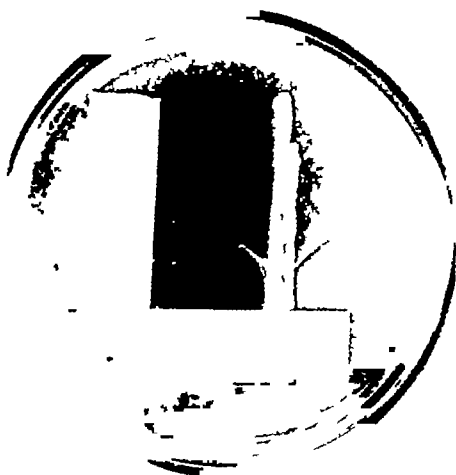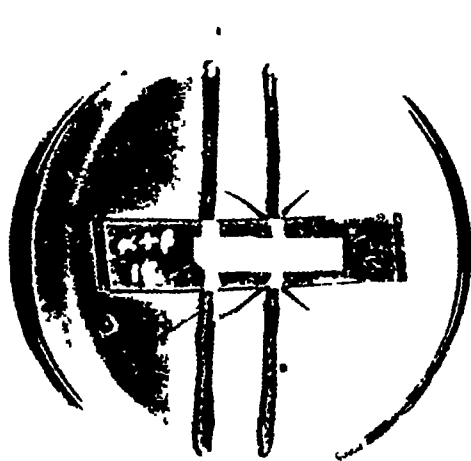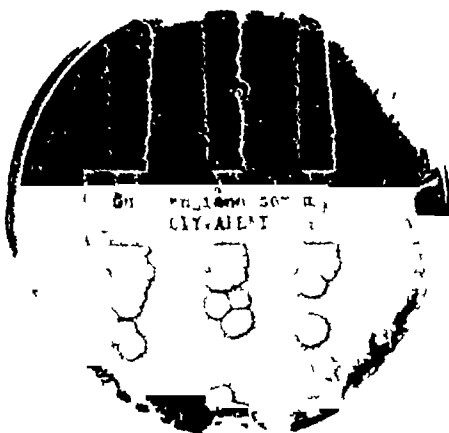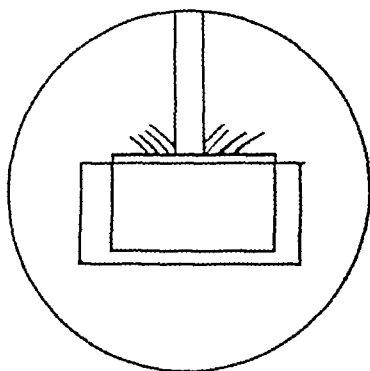
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for various toxoid and antitoxin concentrations are obtained and their distance from the strip edge can be measured From the data thus obtained not only can the antitoxin gradient be plotted, but also the toxoid gradients at various initial values (Fig 5)

This shows that both antigen and antibody can be incorporated in an agar base, so that a concentration gradient develops by diffusion for an appreciable distance The range is theoretically from the original concentration used, at the strip edge, to *nil* at some distance from it Although the actual rate of migration and consequently the shape of the gradiant may be different for
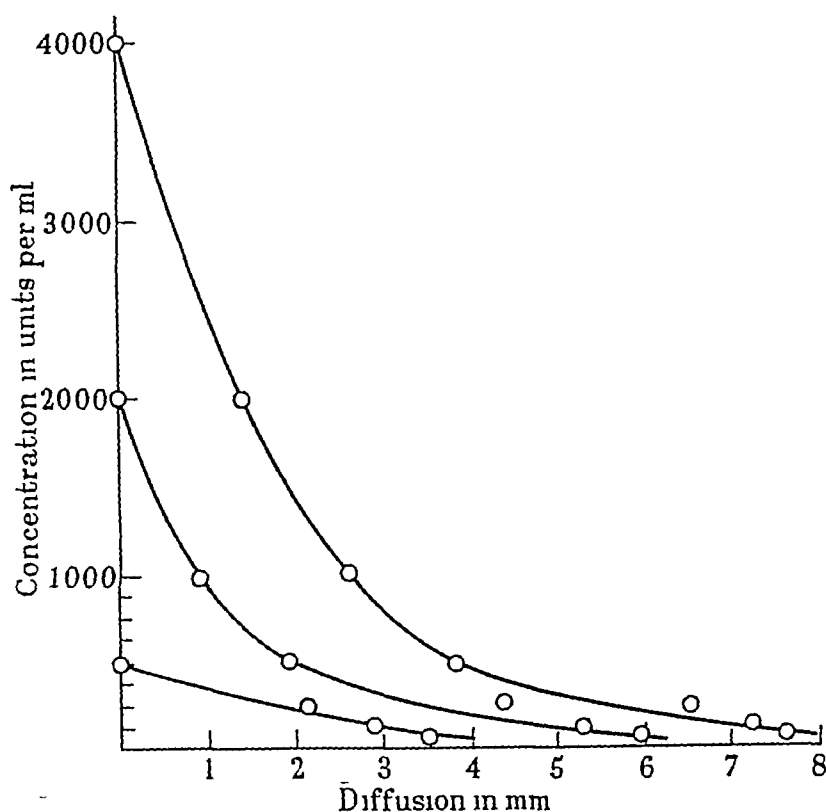
FIG 5 —Diphtheria toxoid diffusion gradients in agar for different initial values after 48 hours' incubation at 37° C

different antigens and antibodies, two gradients set at right angles to each other will lead to flocculation at optimal proportions along a plane which, viewed from above, appears as a line   The fact that what appears to be a line in the photograph is really a plane extending through the entire thickness of the medium can readily be seen in the actual plates if these are viewed by transmitted light when the fine knife-edge of flocculation becomes quite obvious   The lines are seen to be broader at their insertion or may even appear split (see Fig 3a and 3b) because the plane of flocculation is slightly tilted there as already described

The conditions when two diffusion gradients are set at right angles to each other are shown in Fig 6   For the sake of simplification the depth of the medium is disregarded and the diagram reduced to two dimensions   The first set of arrows issuing from the antigen and antibody strips, *a* and *a'* represent their

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concentrations at the end of a certain period of incubation   These concentrations represent equivalence, and at the point of the first pair of arrows precipitation occurs   After a further lapse of time equivalence again occurs at the point of the secondary pair of arrows, *b* and *b'*, although the absolute quantity of the reagents is less   Under these conditions flocculation will occur along a continuous line   In the example chosen the antigen and antibody gradients are such that they have the same shape, and the initial concentration of the two reagents in the filterstrips is equivalent   These conditions produce a straight line passing at 45 degrees between the two strips and starting at the angle formed between them   In flocculation both antigen and antibody leave the system as they pass into the precipitated phase   Along the line therefore their respective concen-
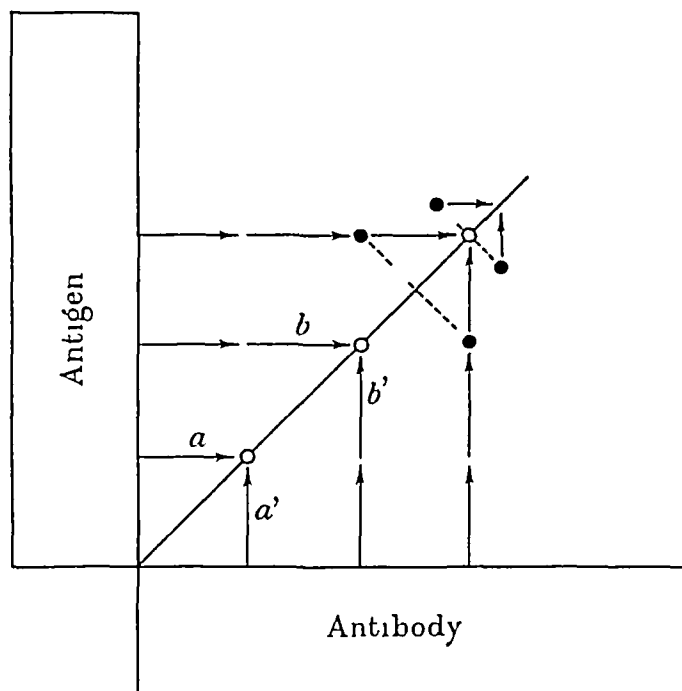
Fic 6 —Diagrammatic representation of the dynamics of the double diffusion gradient

trations are determined by the solubility of the precipitate, and this is for practical purposes *nil*   Particles equidistant from the line, shown in black, will migrate in the direction shown by the arrows, as the gradient has to be reconstituted between the zero concentration on the line and the concentration still existing in the filter-paper   However, the particles taking part in this new migration will also ultimately flocculate along the line   In this way a continuous flow of the reagents occurs towards the line which increases both in thickness and length   Thus the double diffusion gradient is not only free from the zone effect but the dynamic conditions are such that the reaction proceeds continuously towards completion   When the initial concentrations of the reagents in the strips are not equivalent, or the gradients have a different shape, the line of flocculation will not pass through the angle of the strips or the line will not be straight   These conditions are a little more difficult to visualize, but the dynamics of the system remain essentially unchanged

So far only theoretically pure flocculation systems have been considered, 1 e containing a single antigen and a single antibody From the point of view of flocculation, however, a " pure " antigen and a mixed antiserum, containing amongst others the homologous antibody, will still represent a pure system Likewise with a mixture of antigens and a single antibody directed against one of the antigens a pure system results in the sense that the combination of a single pair of reagents is believed to occur These conditions are, however, seldom encountered in practice Antigens used for the preparation of sera are biological products, and even though they may be purified for the flocculation reaction,
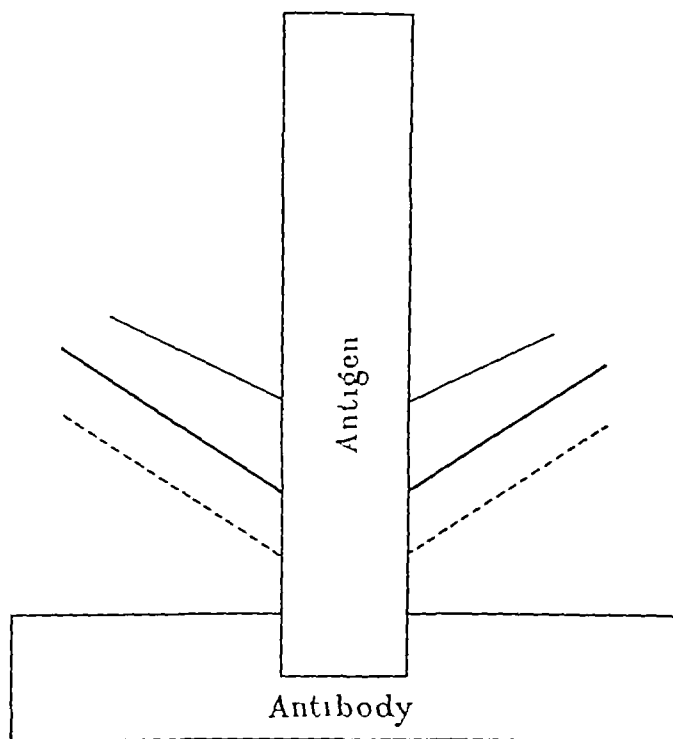
FIG 7 —Resolution of mixed precipitating systems by the double diffusion gradient

physico-chemical purification may not represent serological purity Furthermore even against a theoretically pure antigen several antibodies are possible The utilization of the double diffusion method will usually lead to a separation of mixed systems Fig 7 shows the results with three hypothetical systems The upper thin line, the middle thick line and the lower dotted line represent flocculation reactions Each pair of reagents will produce its own diffusion gradients and will flocculate at its own optimal ratio The conditions of the experiment militate against the overlap of the flocculation lines, as the position, shape and angle of each line will depend on the concentration and diffusion rate of the reagents involved Although these three systems might show up as a broad zone by the tube method, the introduction of diffusion brings a new element which allows each to develop individually Admittedly an overlap of the lines at their insertions may occur even under these conditions but separation will occur further out when the differentiating effect of the diffusion comes into full play To obtain complete overlap of two systems the same initial concentrations

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optimal proportions and diffusion velocities must be postulated for both antigens and both antibodies, and this is not likely to occur

In these remarks concerning the separation of the lines it is assumed that the second stage of precipitation reactions is specific   The numerous examples of multiple flocculation systems, examined with the double diffusion gradient technique, confirm this assumption   Topley, Wilson and Duncan (1935) on the basis of agglutination experiments concluded in favour of Marrack's lattice hypothesis which postulates a specific second stage   They suggested that " the interesting implications of Marrack's hypothesis should be given due weight in future attempts to determine the mechanism of those antigen-antibody reactions in which flocculation plays a part "   While with bacteria and red cells the question is comparatively easily approached experimentally, considerable difficulties arise with the identification of white precipitates of different nature   It will be seen that the double diffusion gradient enables one to identify a system with a known one, and thus furnishes striking proof of the specificity of the second stage in a field where proof was still required

In most naturally occurring systems, such as toxin-antitoxin reactions, body fluid antigens, etc , we know that the antigen is a complex made up of several substances, the number of which may not be known   Likewise the serum may, and often does, contain multiple antibodies   The double diffusion gradient is comparable in its action to prism   the constituent systems are separated into individual lines, as are the colours of the spectrum

The Application of the Double-Diffusion-Gradient Method to Toxin Analysis

In recent years it has become increasingly evident that crude filtrates of organisms known to produce toxin may contain more than one poisonous antigenic factor   Thus the term "" toxin " shifted to denote these individual factors, and in some bacterial groups formed the basis of subclassification   The analysis of toxin pattern, although of great interest from the medical and veterinary point of view, is greatly handicapped by the technical difficulties encountered Suitable indicator effects of the individual toxins have to be found, and large numbers of quantitative tests against various reference sera are required before the multiplicity of the factors can be established

Determination of the number of lines

Our knowledge of the toxic pattern is most complete for the clostridia, and especially *Cl welchii* (Oakley, 1943)   It seemed desirable therefore to observe the application of the method in this field   The experiment was carried out blindly, the constitution of the 8 sera used being unknown   The material was kindly provided by Dr C L Oakley, of the Wellcome Physiological Research Laboratories, Beckenham, Kent   Streak inocula of the organisms were used for the toxin gradient, as in this way if satisfactory results were obtained they could more readily be applied to the laboratory identification of the types   The medium used had the following composition   Difco proteose-peptone 2 per cent, lactic acid (B P) 0 035 per cent, maltose 0 15 per cent, agar pulv 2 per cent, pH = 7 6 This medium was tubed in 10 ml quantities, and immediately before being poured was enriched with 0 5 ml of horse serum and 0 5 ml of Fildes' extract   Strips of

filter-paper, 60 mm by 15 mm, soaked in undiluted clostridial antitoxin, were sunk into the middle of the plate before the medium solidified Young broth cultures of the five organisms tested were centrifuged, and heavy streak inocula
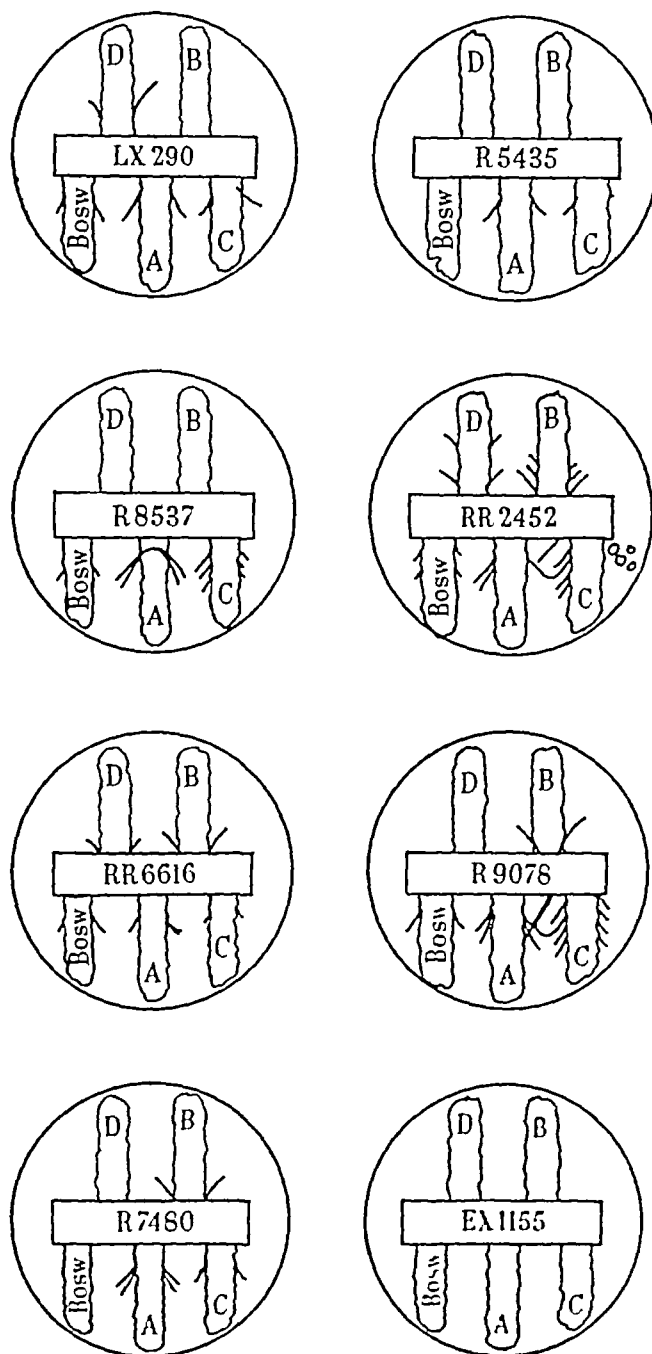
FIG 8 —Flocculation lines produced by different types of *Cl welchii* with various antis ra

made from the sediment in the manner shown in the diagram (Fig 8) The plates were incubated in a McIntosh and Fildes jar for two days and final readings were taken after a lapse of several days at room temperature

The data concerning the sera and toxic patterns were supplied after the conclusion of the experiment, and the information is shown in Tables I and II

TABLE I

Number	Type of organism		α	β	γ	δ	ε	θ	ι	ν	λ
							Toxins				
CN 1491	Cl welchii, Type A, S 107		++	—	—	—	—	+	—	—	—
CN 1990	„ Type B, S 845		(+)	+	+	—	+	+	—	—	+
CN 1797	„ Type C, Strain Ashby		+	+	+	+	—	+	—	+	—
CN 1798	„ Type D, Strain R₂		+	—	—	—	+	+	—	—	+
CN 1241	„ Type Bosworth		+	—	—	—	—	+	+	+	+

TABLE II

Serum	α	β	γ	δ	ε	θ	ι	κ	?
					Units of antitoxin				
LX 290	135	—	—	—	—	0 3	—	140	—
R 5435	0 2	—	—	—	—	190	—	55	—
R 8537	180	—	—	—	—	90	—	180	30
RR 2452	35	1250	—	4	180	45	—	?	?
R 6616	100	—	—	—	200	—	—	?	?
R 9078	75	3100	—	80	<½	700	—	?	?
R 7480	190	—	—	—	—	13	240	?	?
EX 1155	—	—	—	—	—	—	—	—	—

— Denotes lack of a measurable amount of antitoxin   ? Amount not determined

Although the agreement with the expected findings is not absolute, the results are nevertheless striking   The two sera known to contain the highest number of individual antitoxins, R 9078 and RR 2452, give most lines with Cl welchii, Type C, which in turn has the most complex toxin pattern   Serum EX 1155 was a normal human serum and yielded no lines at all   In attempting to assess the agreement, the limitations of the method should be stressed   The medium used may not yield all the toxins obtained in a cooked meat medium, and not all antigenic diffusible products are necessarily toxins   In spite of these limitations one practical application of the method is at once apparent , it seems possible to correlate the number of lines produced with the type of the organism   Thus with suitable modifications a method may be elaborated for the typing of Cl welchii, based on the *number* of lines produced with various test sera

The rule of looping

It follows from the theoretical considerations already discussed that lines produced by identical systems must be continuous by this method, or in other words the lines thus produced must loop   Identical systems for these purposes are those in which either the antigen or the antibody or both are identical   Thus Fig 9 shows looping between the toxoid-antitoxin line issuing from the vertical strip, and the toxin line issuing from a growth of virulent diphtheria bacilli   Although toxoid and toxin differ by their physiological effect showing a difference in chemical constitution, they unite with the same antitoxin and therefore behave like identical systems   Looping occurs because the points of optimum proportions for a single system must fall on a continuous line   As two lines approach each other a summation of the antigen concentration occurs in the region, and the

relatively larger amount of antigen will find its corresponding ratio of antibody nearer to the strip   This will lead to a bending of the lines towards the antitoxin strip and finally to their fusion, since for each antigen concentration there is only one optimal antibody concentration in the field   This only applies, however, if the lines are formed at the same time   Lack of looping—and consequently erroneous interpretation—may occur if one of the lines is already well formed when a second approaches it   The dynamic conditions of the double diffusion gradient are such that the concentration gradients suffer a deformity in the vicinity of a line after a while, and consequently the second line approaching it may show angular joining or none at all

Looping is a practical means of identifying an unknown system with a known one   Furthermore, in the case of multiple lines produced by different strains looping will show whether the diffusible antigens are shared or not   Fig 10 shows two strains of staphylococci tested against a serum known to be rich in antibodies to alpha and beta haemolysins   The medium used was Difco heart-infusion broth to which 0 2 per cent KH_2PO_4, 0 03 per cent $MgSO_4$ and 1 5 per cent agar were added, and the pH adjusted to 7 4   Plates were poured with the addition of 1 per cent horse red cells washed in saline, reconstituted and laked with saponin   The plate was incubated in 30 per cent CO_2 in air for two days, and photographed after four days at room temperature   Amongst others two heavy lines are issuing from strain " W 46 "   The antigen responsible for one of these is not produced by strain " CN 338 " and the line cuts across the streak of growth   Looping is shown by the other lines, revealing that these antigens are shared   Looping can thus be made use of in various ways   The diffusible antigen pattern, which includes the toxins, of organisms can be studied and compared   The individual systems can be further investigated by means of filterstrips soaked with known fractions of toxic filtrates

The rule of overlap

Some toxins have visible indicator effects which can be demonstrated in plates, e g various haemolysins, lipase, lecithinase and other turbidities   As the proportions for neutralization of a toxin and the optimal proportions for flocculation show close agreement, the indicator effect would be expected to stop abruptly short of the flocculation line   On one side of the line there is free toxin, capable of producing the indicator effect, and on the other side there is none   In fact it has been found that the edge of a visible indicator effect and the flocculation line coincide on the double-diffusion-gradient   This may be used for the identification of the toxin responsible for a line   Fig 11a shows three strains of Cl welchii, Type A, against commercial gas gangrene antitoxin on Hayward's (1943) medium, but without the addition of human serum   Fig 11b shows the same experiment, but this time with human serum added   Two lines associated with each strain are visible on both of these plates, and they are probably due to the alpha and theta toxins   The boundary of the Nagler effect coincides with the lines further from the filterstrip, showing that these were the lines produced by the alpha toxin

The method may find useful application when several haemolysins are produced by an organism, all acting on the red cells of the same species   Fig 12 shows a sheep blood agar plate with a strain of staphylococcus   At least three different staphylococcal haemolysins are known to act on sheep red cells   The

strain shown produces a wide zone of haemolysis which is completely inhibited, but between it and the filterstrip another haemolysin shows which is only partially inhibited   As the serum used was known to have a high anti-alpha titre, it may be assumed that the line demarcating the wide-zoned haemolysis, which is further from the strip, is that of the alpha haemolysin

The specific nature of a turbidity appearing on a plate may also be readily investigated by the double diffusion gradient method   Thus it was observed that some staphylococci grown aerobically on agar media containing laked horse red cells produce a marked turbidity around them   Fig 13 shows four strains tested against a staphylococcal antitoxin   (The negative and positive signs refer to previous coagulase testing)   The first strain, 10381, shows no turbidity The coagulase negative strain shows heavy turbidity around it but it is not inhibited by the antitoxin   The coagulase positive strain shows no turbidity, showing that the effect is independent of coagulase   Strain " 6, however, shows a turbidity which is inhibited in the same wedge shape as were the Nagler effect and the alpha haemolysin shown in the previous pictures   Under the condition of the experiment at least two turbidity factors appear therefore, one which is inhibited by the antitoxin and one which is not   The former appears to be a hitherto undescribed indicator effect of one of the diffusible antigenic products of staphylococci

The Application of the Double-Diffusion-Gradient to Serum Antigens

Serum antigens generally yield broad zones with the tube method commonly employed, and also show the anomaly of different optima with the constant antibody (alpha) and the constant antigen (beta) titrations   As both these findings were considered to reflect the basic flocculative behaviour of such systems, it is of interest that multiple thin lines, similar to the toxin-antitoxin lines can be obtained with serum antigens using two diffusion gradients set at right angles The multiplicity of antigenic factors in sera is a fact well known from physico-chemical studies, but serological discrimination is likely to go yet further   Oudin (1947) incorporated rabbit serum prepared against horse serum in an agar base, and overlayered it with the antigen and various chemically separated fractions of it   Multiple planes of flocculation appeared, as in the case of Bechhold s experiments, and these were interpreted as representing serological fractions In this type of experiment the two diffusion gradients are set in opposition to each other, i e at 180°, and the dynamic conditions resulting are unsatisfactory As flocculation proceeds the ratio of antigen to antibody on either side of the plane of flocculation becomes altered   Finally a gross excess of one of the reagents will cause the floccules to dissolve, and the plane of flocculation will appear to move on   The planes thus produced are fuzzy and difficult to observe for prolonged periods   When the two diffusion gradients are set at 90°, however, the planes of flocculation are well defined and stationary as the dynamics of the system produce a continuous and progressive reaction

The difficulty in setting up a right-angled double diffusion gradient, similar to the one used in toxin experiments, is in bringing enough antibody into the system   When a serum is used for immunizing, vastly different titres of antibody may appear against the different antigens contained in it   The titre usually determined refers merely to the major antigenic component   An antiserum with an apparently high titre therefore may be deficient in antibodies against the other

antigens, and to reveal their presence an increased concentration is required Various means can be used to increase the amount of antibody in the gradient One method is shown in Fig 14, and may be described as a " feeder plate " It shows the behaviour of human serum against a rabbit anti-human serum The plate was poured with 2 per cent agar with 0 5 per cent salt (not nutrient agar, to reduce contamination), and a wide piece of filter-paper was embedded *vertically* and held in position until the agar set A microscope slide cut down in length was placed adjacent to it and the strip bent over it Several drops of anti-human precipitating serum were placed on the strip, the glass slide underneath preventing the antibody from leaking on to the plate Finally a narrow strip of filter-paper soaked in human serum was placed at right angles to the first strip on the surface of the agar The plate was incubated for three days, further drops of antibody being placed daily on the free edge of the strip This arrangement produces a good antibody gradient with a weak serum The wide strip is acting as a wick, and the total amount, rather than the concentration, of antibody entering the gradient is increased The diagram shows the development of two heavy, two fine, and one very fine line, suggesting that there are at least five different systems The individual existence of these cannot be demonstrated by the tube method

Another technique is to increase the antibody concentration in the strip by drying-in successive amounts Fig 15 shows the application of this method Three filterstrips were repeatedly soaked with the antibody and dried *in vacuo* In this way an increased volume of antiserum was incorporated in the strips and the initial concentration raised The three strips superimposed were sunk into a salt-agar matrix This arrangement obviates the objection of intermittent feeding with antibody and the possibility of producing waves Human serum was separated into two fractions using Pillemer and Hutchinson's (1945) methanol technique The vertical strip shown on the left of the picture was soaked in the " albumen " fraction, the middle in normal human 'serum, and the one on the right in the "globulin" fraction The plate showed an almost identical pattern with normal serum and the globulin fraction, while the albumen fraction showed only a fine haze This particular anti-human serum appears to be predominantly anti-globulin

Methods utilizing the right-angled double diffusion gradient principle offer possibilities for the investigation not only of antigen mixtures, but also of different antibodies Nor is the method limited to toxin or serum-antiserum systems Recently it was found possible to demonstrate the presence of at least two impurities in a highly refined preparation of diphtheria toxoid and this finding was in good arrangement with the electrophoretic pattern, which also showed two added substances

DISCUSSION AND SUMMARY

In the foregoing an approach to the characterization of macromolecules capable of acting as antigens or haptens has been described It is based on the combination of diffusion and specific serological flocculation The principle is to place two diffusion gradients at right angles to each other It is claimed that under these conditions flocculation will occur at optimum proportions under dynamic conditions, and the reaction will tend to move towards completion These conditions will be free from some of the limitations and disadvantages of

the tube method commonly used in serology   Furthermore the arrangement described reveals the nature of complex flocculating systems by spatial separation of the individual antigen-antibody reactions, and thus yields a new method for the investigation of homogeneity

The examples illustrating the use of the double-diffusion-gradient were drawn chiefly from the field of bacterial toxins, where its usefulness is most immediately obvious   With suitable adaptations, involving the design of the matrix for high toxin yields, the method may be used for the detection of toxin-producing bacterial strains in routine work   It is also capable of extension to the analysis of multiple toxins, and the toxin-pattern of strains can be investigated by this relatively simple method

The double-diffusion-gradient is essentially a qualitative approach effecting only a separation of the individual systems   The agar technique described in Petri dishes is, however, only an application of the principle, in the same way as filter-paper chromatography is an application of chromatography itself   The different planes of flocculation should be capable of production on a larger scale than possible in the shallow layer of a Petri dish   These planes of precipitation could be physically separated and further analysed, as they are likely to represent a purer combination than the floccules obtained by the tube method

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SPONTANEOUS MUTATION TO STREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI*

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MANY theories have been advanced to explain the increase in resistance of bacteria which frequently follows their exposure to an antibiotic   The view that the action of the antibiotic is selective, killing all but resistant bacteria which have arisen by mutation from sensitive bacteria, is now widely accepted   The critical experiments of Luria and Delbruck (1943), which showed that virus resistant bacteria arose by mutation from sensitive bacteria independently of the action of the virus, supports this view   Studies of the type carried out by these authors give not only quantitative information about the incidence of variant bacterial strains, but also provide a means of studying the problem of mutation in living cells   In view of this it seemed worth while to apply the methods of Luria and Delbruck to another strain of the bacterium they studied, *Escherichia coli*   Consequently tests were carried out to determine—

(1) Whether *E coli* highly resistant to streptomycin occurred in cultures which had not been exposed to streptomycin

(2) Whether resistant colonies retained their resistance on subculture and were all of the same type

(3) The mutation rate from sensitivity to full resistance

Other aspects of this general problem are being investigated in this laboratory by Newcombe and Harwirko

MATERIALS AND METHODS

Materials

Escherichia coli strain B/r (Witkin, 1946, 1947) was the organism studied, all test cultures being inoculated from a single bacterial suspension

Two media, Difco nutrient broth with sodium chloride added to a concentration of 5 per cent and a synthetic medium (M₉ of Anderson, 1946) were used for test cultures

For testing resistance, 10,000 µg of streptomycin sulphate (Lilly) were spread on 10 ml of agar in Petri dishes   These were stored overnight at 5° C to permit diffusion   The final concentration of streptomycin averaged 1000 µg per ml

Experimental procedure

The procedure was a modification of that used by Luria and Delbruck   Eight or ten cultures, started from inocula so small (100 to 1000 bacteria) that there was little chance of a resistant bacterium being included, were incubated for 48 hours

With all M_9 cultures, air was bubbled through the tubes during the last 24 hours
in order to increase the population   The incubation temperature for plates and
cultures was in all cases 37° C

The final numbers of bacteria were estimated from plate counts   In the case
of the resistant bacteria a suspension of the centrifuged culture, complete except
for the small sample (0 1 ml) taken for the total assay was plated on strepto-
mycin agar   The large number of bacteria plated, formed a milky film which
remained unchanged during 48 to 72 hours' incubation except for the development
of colonies on certain of the plates

A representative number of the surviving colonies were suspended in sterile
saline and tested for resistance in the following manner   Equal quantities of the
suspension were plated on plain and streptomycin agar, incubated and colony
counts made   The resistant colonies fell into two categories

    (1) *Resistant* independent (*r*), (colonies on both plates)
    (2) Resistant *dependent* (*d*) (colonies on streptomycin containing agar only)
    The terms *resistant* and *dependent* will be used for these

Calculations

All calculations are by the methods of Luria and Delbruck (1943)   These
include a test of the hypothesis that resistant bacteria arise by mutation and the
calculation of the mutation rate (a) *the probability of occurrence of a mutation per*
bacterium per bacterial generation   Two methods are available for the latter,
one which uses the proportion of cultures with no resistant bacteria (p_0) and one
which uses the average number of resistant bacteria per culture (r)   Values of
the total population (N_t) and the number of test cultures (C) are also needed
The three formulae are as follows, Formulae 1 and 2 being in the form given by
Newcombe (1948)

    (1) $a = -(\log_e 2)(\log_e p_0)/N_t$
    (2) $r = (a N_t/\log_e 2) \log_e (C a N_t/\log_e 2)$
    (3) $\text{Variance}_r = C a^2 N_t^2$

<div align="center">EXPERIMENTAL</div>

Sixty cultures tested in the above manner are described in Table I   Twenty-
four of them contained bacteria which produced colonies on streptomycin

Twenty of the resistant colonies were then tested in the manner described
Of these, fourteen were *resistant* and five *dependent* (Table II)   *Resistant* and
dependent strains have now been observed in many normally sensitive species
(Miller and Bohnhoff, 1947 ; Paine and Finland, 1947 ; Yegian and Budd, 1948
Spendlove, Cummings, Fackler and Michael, 1948 ; Hobby and Dougherty,
1948 ; Alexander and Leidy, 1947)

A variant from the parent strain which arises spontaneously and passes on
its new characteristics to succeeding generations, is termed a mutant   Further-
more, as has been pointed out by Luria and Delbruck (1943), variants which are
mutants are likely to be distributed throughout a series of small cultures with
considerable culture to culture difference in frequency   The present series with
one giving rise to 815 resistant colonies , three to more than 20 , the remainder
to less than 10, displays this characteristic   Values of a function of this *variance*

TABLE I —*The Frequency of Occurrence of Resistant Bacteria in Cultures of Escherichia coli Strain B/r (Witkin, 1936)*

Experiment	A.	B	C	D	E	F	G
Number of cultures	10	10	8	8	8	8	8
Culture medium	Broth	Broth	M_9	M_9	M_9	M_9	M_9
Volume of culture (ml)	10	10	10	10	10	10	10
Average number of bacteria in inoculum	?	8	161	210	126	86	78
Average end number of bacteria per culture, $\times 10^9$	2 4	1 9	32	22	29	20	26

Numbers of resistant bacteria in individual cultures							
	1	0	0	0	0	0	0
	0	2	0	4	1	0	0
	0	0	0	4	1	0	0
	0	0	3	3	6	0	0
	1	4	0	2	2	2	0
	0	0	815	0	1	0	40
	0	27	9	7	0	0	2
	0	0	0	8	0	29	3
	0	0					
	0	0					

Data concerning mutation to resistance							
p_0	800	700	625	250	375	750	625
r	2	4 1	103	3 5	1 4	3 9	5 6
Rate (Formula 1) $\times 10^{-11}$	6 4	13 0	1 0	4 4	2 4	1 0	1 3
Rate (Formula 2) $\times 10^{-11}$	6 8	45 7	42 2	4 4	1 8	5 3	5 3

$\dfrac{\text{Stand dev}}{r}$ { exper	2 00	1 96	2 61	0 78	1 34	2 44	2 33
calc	2 58	0 67	0 37	0 78	1 06	0 77	0 70

in the number of mutants per culture (the *standard deviation* divided by the *mean*) are given in Table I These may be compared with the value of the same function to be expected if the resistant cells arise by mutation (Formula 3) The present data confirm those of Luria and Delbruck (1943) in that the experimental value is even greater than that predicted It is therefore suggested that the resistant colonies are descended from resistant cells which have arisen by mutation in the test cultures Alexander and Leidy (1947) take a similar view of their streptomycin resistant strains of *H influenzae* Type b

The estimated values of the mutation rate (Table I) range from 1 $\times$ 10^{-11} to 13 $\times$ 10^{-11} by Formula 1, with an average of 4 4 $\times$ 10^{-11} , and from 1 8 $\times$ 10^{-11} to 45 7 $\times$ 10^{-11} by Formula 2 with an average of 16 $\times$ 10^{-11} The value by Formula 1 is generally the lower A difference of similar magnitude and in the same direction was also observed by Luria and Delbruck (1943) Demerec and Fano (1945), and Newcombe (1948), and this has been shown by the latter to be due to a downward bias with Formula 1 It is therefore likely that the higher

TABLE II —*Assays of Streptomycin Survivors*

Colony tested	Plate counts		Classification of tested colony *
	Agar	Agar and strept	
1	91	121	r
2	360	320	r
3	17	20	r
4	0	12	d
5	2139	2429	r
6	169	190	r
7	83	74	r
8	2549	3100	r
9	1345	1374	r
10	15	19	r
11	0	99	d
12	0	45	d
13	157	151	r
14	0	492	d
15	142	0	s
16	0	184	d
17	35	59	r
18	184	142	r
19	9	13	r
20	492	263	r

r = resistant , d = dependent , s = susceptible

value by Formula 2 is nearer the truth But the latter formula is strictly applicable only when growth rates of parent and mutant strains are the same, a condition which is unfulfilled whenever *dependent* bacteria are present for, according to Newcombe and Hawirko (1949), such bacteria undergo at the most only two divisions in streptomycin free broth Consequently values by this formula also suffer from a downward bias and it is likely that the mutation rate is higher than 16×10^{-11} and that a resistant bacterium will arise at the division of a sensitive one when the population approaches ten thousand million This is of the same order as the mutation rate to streptomycin resistance in *H influenzae* Type b (4×10^{-11}) (Alexander and Leidy, 1947), and lower than that to phage resistance in *E coli* (1×10^{-8} or higher) (Demerec and Fano, 1945)

DISCUSSION

The foregoing data are consistent with the view that resistance to streptomycin in *E coli* strain B/r arises as the result of the mutation of sensitive bacteria Consequently resistant bacteria will appear with a characteristic frequency (the mutation rate) in susceptible populations independently of the presence of streptomycin Once such a mutation has taken place, the bacterial population will contain at least one bacterium capable of growth in the presence of streptomycin Addition of streptomycin will destroy the susceptible bacteria, leaving the resistant ones and this will not be remedied by further administration of streptomycin Withdrawal of streptomycin on the other hand may be destructive to the resistant

population if the bacteria are of the dependent type To destroy independent ones an antibiotic other than streptomycin must be sought

Reports of research in which streptomycin has been given in combination with another substance are of special interest in this connection Smith, McClosky, Jackson and Bauer (1947) report greater recovery from tuberculosis in guinea-pigs when treated with streptomycin and a sulphone compound than when treated by either substance alone Encouraging results were obtained by Lincoln (1948) following treatment of patients suffering from tuberculous meningitis and miliary tuberculosis with streptomycin and promizole A study of the effect of other antibiotics on the resistant strains should be of value in discovering additional combinations of therapeutic value

SUMMARY

(1) *Escherichia coli* strain B/r resistant to 1000 micrograms per ml of streptomycin can be isolated from populations of the order of 1×10^{10} bacteria by plating in the presence of the drug

(2) These are of two types, one of them requiring streptomycin for growth, the other not

(3) The highly variable distribution of these resistant bacteria throughout the series of cultures examined is consistent with the view that they arise by mutation

(4) The mutation rate for the change from susceptibility to resistance is of the order of 1×10^{-10} per bacterium per bacterial generation

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BLOOD HISTAMINE IN THE ANAPHYLACTOID SHOCK PRODUCED BY ANTI-PLATELET SERUM IN DOGS [*]

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The work of Dale and Laidlaw (1919) and Dragstedt and Gebauer-Fuelnegg (1932) showed histamine to be the aetiological agent responsible for anaphylactic shock, this substance being found in sufficient concentration in the blood to explain the majority of the observed signs. High levels of blood histamine were also found in peptonic (Dragstedt and Mead, 1937) and tryptic (Arellano, Lawton and Dragstedt, 1940) shock.

It was recently shown by one of us (Cruz and Silva, 1949) that the classical symptoms of the anaphylactic reaction could be produced in guinea-pigs, rabbits, rats and dogs by the intravenous injection of the appropriate anti-platelet serum. This phenomenon has been submitted to more detailed analysis, and this paper presents the results of experiments designed to study the variations of the blood histamine concentration during anti-platelet shock in dogs.

MATERIAL AND METHODS

Dogs, previously anaesthetized by thionembutal, were used for these experiments. The carotid blood pressure was recorded by a mercury manometer and samples of blood for histamine estimation were taken either from the jugular vein or femoral artery three times in each experiment—before the injection of the anti-platelet serum, after the injection of the serum when the blood pressure fell, and 8 to 15 minutes after the blood pressure reached its lowest level. It was sometimes impossible to take this last sample owing to the low level of the blood pressure or the death of the animal. The concentration of histamine in the blood was determined by the method of Code (1937). The amount of anti-platelet serum injected varied with its titre according to Tocantins' method of dosage (1936), the dose usually varying between 2 and 4 c.c. per kg. body-weight, corresponding to titres of serum 1.16 and 1.32. The histamine concentration was expressed in terms of histamine base.

RESULTS

The blood concentrations found are shown in Table I.

TABLE I —*The Histamine Content of the Blood of the Dogs before and after the Injection of Anti-platelet Serum*

Number of dog	Histamine before injection of anti platelet serum (μg per c c)	Histamine 2 min after injection of anti-platelet serum (μg per c c)	Histamine 8–15 min after injection of anti platelet serum (μg per c c)
1	0 133	0 120	—
2	0 150	0 133	0 086
3	0 160	0 185	0 172
4	0 030	0 030	0 036
5	0 207	0 256	—
6	0 240	0 300	—
7	0 073	0 070	—
8	0 150	0 135	0 042

The figures in Table I show that there was no significant difference between the blood histamine level before and after the injection of anti-platelet sera In no experiment was a discharge of histamine observed such as is encountered in anaphylactic and peptonic shock The injection of the antisera even caused death in some of the animals without there being any significant variation in the blood histamine concentration These results show that anti-platelet shock in the dog is not due to the liberation of histamine

CONCLUSIONS

The anaphylactoid shock produced in the dog by the injection of anti-platelet serum was not accompanied by any change in the concentration of histamine in the blood

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SURVEY OF PAPERS

HUMPHREY finds that the titre of antistreptolysin S in sera of man and other species is greatly reduced by treatment with ether or alcohol-ether although known antibodies are little affected Antistreptolysin S activity is a complex phenomenon mainly dependent on labile lipoprotein components associated with the albumen fraction (p 365)

ALDRIDGE, BARNES AND DENZ find that beryllium injected intravenously in rabbits, rats and mice as solutions of ionized salts in doses of 0 5 to 1 mg per kg causes death from acute liver necrosis Beryllium ions become rapidly attached to protein and are carried in this form to the liver after intravenous injection, but after subcutaneous injection most of the beryllium remains attached to tissue proteins producing local inflammatory changes (p 375)

ENNOR finds that injections of extracts of the anterior pituitary gland cause an increase in fat and an increase in oxygen consumption and acetoacetate production in the liver of the guinea pig The results are compared with those obtained in the fatty liver following treatment with carbon tetrachloride (p 389)

ENNOR AND SINGER find that the increase in liver fat following injection of anterior pituitary gland extract in guinea-pigs can be prevented by the administration of immune serum prepared by injecting rabbits with anterior pituitary extract (p 395)

MARSTON, using the Craig counter current partition method, has isolated three distinct antibiotics, proactinomycins A, B and C, from the antibiotic proactinomycin (p 398)

MARSTON AND FLOREY find that proactinomycins A and C have about the same antibacterial activity while proactinomycin B is about half as active as the other two In view of their low antibacterial power relative to toxicity it is unlikely that any of the three substances would be useful clinically (p 407)

BURNET, STONE, ISAACS AND EDNEY find that influenza A virus strains in the true O phase as isolated from human subjects cannot be induced by any manipulations in vitro to agglutinate fowl erythrocytes The appearance of the D phase, which agglutinates fowl cells, during passage in the chick embryo is thus due to processes of mutation and selective survival (p 419)

ARRIAGADA, SAVAGE, ABRAHAM, HEATLEY AND SHARP describe the production in potato dextrose broth of an antibiotic from a strain of B licheniformis This organism was originally called A 5 and the antibiotic has been named ayfivin (p 425)

HILLS, BELTON AND BLATCHLEY have devised a synthetic medium for the production of the antibiotic, ayfivin, by B licheniformis On a similar medium a different antibiotic is produced with licheniformin-like characteristics (p 427)

SHARP, ARRIAGADA NEWTON AND ABRAHAM describe the process of extraction of the antibiotic ayfivin, from culture fluids and its further purification Ayfivin is quite different from licheniformin but appears to be similar to bacitracin (p 444)

ARRIAGADA, FLOREY, JENNINGS AND WALLMARK find that ayfivin has powerful antibacterial properties and low toxicity to mice It can protect mice against experimental infection with Str pyogenes and Staph aureus (p 458)

ELEK shows that, when two strips of filter paper are soaked in solutions of antigen and antibody respectively and embedded at right angles to each other in a shallow layer of agar, a line of flocculation appears with a single reacting system and multiple lines appear with a multiple system. He discusses the uses of this technique (p 484)

SCOTT finds that the mutation rate from susceptibility to resistance to streptomycin of a strain of *Bact coli* is of the order of 1×10^{-10} per bacterium per bacterial generation (p 501)

MOUSSATCHÉ AND CRUZ find that the anaphylactoid shock produced in the dog by the injection of anti platelet serum is not accompanied by any change in the concentration of histamine in the blood (p 506)

AUTHORS' ERRORS

P 244, the two curves A and B of Fig 2 have been reversed

P 253, line 29   For Fig 2 read Fig 1

P 286, line 19   For (Fig 1 and 2) read (Fig 1)

„   line 30   For (Fig 3 to 8) read (Fig 2 to 8)

STUDIES ON THE NUTRITION OF *PASTEURELLA PESTIS*, AND FACTORS AFFECTING THE GROWTH OF ISOLATED CELLS ON AN AGAR SURFACE

D HERBERT

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THE work to be described in this paper arose out of an attempt to develop an accurate viable count procedure for *Pasteurella pestis* It was desired to adopt the technique of Miles and Misra (1938), in which standard drops of a suitably diluted suspension are delivered on to the surface of agar plates, which are subsequently incubated and counted A medium and growth conditions were required which allowed every isolated organism on an agar surface to develop into a colony , for *Past pestis* these requirements are not satisfied by ordinary media

Schutze and Hassanein (1934), in a study of the same problem, found that heavy inocula of *Past pestis* would grow on the surface of ordinary agar plates, but widely separated single cells would not grow Growth of single cells could be obtained by adding to the agar either whole blood, pepsinized blood, or extracts of various bacteria Alternatively, isolated cells would develop on ordinary agar if the plates were incubated anaerobically

These findings were confirmed, and attempts were made to discover the nature of the substance present in blood that is responsible for this effect, and why it is only required under aerobic conditions It was also necessary to study the nutrition of *Past pestis* and its growth in chemically defined media While the results of this investigation are by no means complete, they have led to an accurate counting technique for *Past pestis*, and are also thought to be of sufficient interest on their own account for publication at the present time

dl-leucine, dl-isoleucine, dl-norleucine, dl-aspartic acid, dl-lysine, l-arginine, l-histidine, dl-proline, l-hydroxy-proline, dl-phenylalanine, dl-threonine, all $M/1000$, dl-serine, dl-tyrosine, dl-cystine, dl-methionine, all $M/2000$, dl-trypto-phane, $M/5000$ (The above concentrations refer to the final concentration of l-isomer in the medium) All dl-compounds were synthetic The medium also contained KH_2PO_4, $M/30$, $(NH_4)_2SO_4$, $M/100$, $MgSO_4$, $M/5000$, $CaCl_2$, $M/100,000$, $FeSO_4$, $M/200,000$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, all $M/500,000$, glucose, $M/100$, and NaOH to adjust to pH 7 6 The medium was made up and tubed at one and a half times strength, being diluted by the inoculum, other additions and water to make up volume Five ml quantities were incubated in 50 ml Erlenmeyer flasks, in jars containing air/5 per cent CO_2, or H_2/5 per cent CO_2 for anaerobic experiments

This medium contains most amino-acids in rather higher concentrations than are usual in synthetic media, this was found to give heavier growth

Viable counts —Seven drops (0 2 ml) of suitably diluted bacterial suspensions were delivered from calibrated dropping pipettes on to the surface of agar plates, and spread by rotation Plates were incubated at 37°, either in a "humid' incubator or in sealed jars containing a pad of moistened cotton-wool

Dilutions were made in 0 9 per cent NaCl or 1 per cent phosphate buffer pH 7 6, broth is not superior to these simple diluents

Haematin estimations were made by the pyridine-haemochromogen method of Keilin and Hartree (1935) and Rimington (1942)

Strains —Five strains of *Past pestis* were available Three of them (No 327, 336 and 337) were highly virulent (Herbert, 1947), they had been preserved by drying soon after primary isolation from human plague cases in N Africa Strains "Soemedang" and "Tjiwidej" were the completely avirulent "live vaccine' strains of Otten (1936) Stocks were preserved in the dried state by the gelatin-ascorbic acid technique of Stamp (1947), cultures in current use were grown in peptic blood agar stabs and kept at 0°, subcultures being made monthly for not more than six months

RESULTS.

Necessity of a substance present in peptonized blood for aerobic growth of isolated cells

The basic phenomenon under investigation is illustrated in Table I and Fig 1 Two series of Evans' peptone-agar plates, to one of which pepsinized sheep s blood had been added, were seeded with serial dilutions of an 18-hour broth culture of *Past pestis*, and incubated aerobically at 37° With high dilutions (less than 650 cells per plate) no colonies developed on the plain agar plates in 40 hours, and less than 6 per cent of the expected number developed even after 5 days' incubation, on the plates with added peptic blood, however, all the colonies were fully developed in 40 hours (and were just visible in 24 hours) With very heavy inocula producing confluent growth there was no visible difference between the two series The peptic blood, therefore, is only necessary for the develop-ment of widely isolated single cells

Table II shows that under *anaerobic* conditions peptic blood is not required, and the full number of colonies develops on plain agar Under semi-anaerobic conditions, as in poured plates incubated in air, a proportion of the cells develops in plain agar, but not the full number Plates containing 25 ml of agar were used, and it could be seen that colonies only developed deep in the agar Under

TABLE I —*Effect of Pepsinized Sheep's Blood on Growth of Small Inocula on Agar Plates*

Dilution of broth culture , 1 in—	Evans peptone Colonies visible in—		Evans peptone plus 5% pepsinized sheep's blood Colonies visible in—	
	40 hours	5 days	40 hours	5 days
10	Confluent	Confluent	Confluent	Confluent
4 × 10⁵	0	33	ca 600	Semi-confluent
8 × 10⁵	0	19	324	324
16 × 10⁵	0	7	148	148
32 × 10⁵	0	3	70	70
64 × 10⁵	0	3	35	35

Evans peptone agar plates, with or without pepsinized sheep's blood, seeded with 7 standard drops (0 2 ml) of the indicated dilution of an 18-hour broth culture of strain " Tjiwidej," and incubated aerobically at 37°

TABLE II —*Effect of Pepsinized Blood in Surface and Poured Plates under Aerobic and Anaerobic Conditions*

		Colonies visible in 40 hours	
		Evans peptone	Evans peptone + 5% pepsinized blood
Aerobic	Surface plates	0	375
	Poured plates	34	343
Anaerobic	Surface plates	362	354
	Poured plates	352	369

All plates seeded with 7 standard drops (0 2 ml) of a suspension of strain ' Tjiwidej and incubated at 37°, either in air or in a McIntosh and Fildes anaerobic jar

anaerobic conditions the colonies are smaller, more flattened, and take longer to develop than those produced aerobically in the presence of peptic blood (Fig 1) but the same number of colonies develops in both cases

In ordinary media therefore, *Past pestis* behaves as if it were an anaerobe while in the presence of pepsinized blood it grows aerobically or anaerobically

Identification of the active substance in pepsinized blood

The nature of the substance in peptic blood responsible for the above effects was now investigated Fildes (1920 1921) who first used peptic blood for the cultivation of *Haemophilus influenzae*, showed that its growth-promoting effect was due to two substances, (1) haematin, and (2) a heat-labile factor, later named " V-factor " and eventually identified as triphosphopyridine-nucleotide It was found that autoclaving the peptic blood did not affect its growth-promoting action for *Past pestis*, which immediately suggested that the active substance might be haematin Small amounts of haemin were then added to the basal agar medium and were found to promote the aerobic growth of isolated cells in exactly the same way as peptic blood (Tables III to V) Commercial samples of haemin and a highly purified specimen had quantitatively identical effects

These experiments alone do not prove that the effect of the peptic blood is entirely attributable to the haematin it contains To test this point the h...

content of a sample of peptic blood was determined by the pyridine-haemochromogen method, and a series of plates containing falling concentrations of peptic blood was compared with a second series containing equivalent amounts of pure haemin   The results (Table III and Fig 1) show that the effect of peptic blood can be entirely accounted for by its haematin content   In both series 100 per cent of colonies developed with 5 µg haemin/ml , and ca 15 per cent developed with 1 µg /ml , with sub-optimal amounts of haemin or peptic blood the colonies were irregular in size as well as deficient in numbers (Fig 1)

With large amounts of peptic blood (>2 per cent) colonies are visible earlier, and are definitely larger after 40 hours than with equivalent amounts of pure haemin, though the same number of colonies develops   This is probably due to the presence in peptic blood of other substances having a generalized accelerating effect on growth   with lower concentrations of peptic blood the effect is hardly noticeable

TABLE III —*Comparison of Pepsinized Sheep s Blood and Equivalent Amounts of Haemin*

Additions to basal medium	Colonies developing in 40 hours
Haemin 10 µg /ml	141
,, 5 ,,	149
, 2 ,,	55
, 1 ,,	29
, 0 5 ,,	2
1% pepsinized sheep blood (10 5 µg haemin/ml)	146
0 5% ,, ,, ,, (5 25µg ,,)	134
0 25% ,, ,, ,, (2 01 µg ,,)	107
0 1% ,, ,, ,, (1 05 µg ,,)	17
0 05% ,, ,, ,, (0 525 µg ,,)	1
No additions	0
,, grown anaerobically	138

Basal medium tryptic meat agar, containing the indicated addition of pure haemin or pepsinized sheep's blood of known haemin content   All plates seeded with 7 drops (0 2 ml) of the same suspension of strain " Tjiwidej " and incubated at 37° in air, or in anaerobic jars

TABLE IV —*Effect of KCN and Haemin under Aerobic and Anaerobic Conditions*

	Number of colonies developing in 40 hours			
	Aerobic		Anaerobic	
KCN conc (M)	No haemin	Haemin 10 µg /ml	No haemin	Haemin 10 µg /ml
0	0	370	340	350
0 0002	0	186	348	356
0·001	0	36	402	351
0 004	0	0	385	395
0 02	0	0	325	327

The basal medium was tryptic meat agar, containing the indicated additions of haemin or KCN (neutralized)   All plates seeded with 7 standard drops (0 2 ml) of a suspension of strain " Tjiwidej," and counted after 40 hours' incubation at 37°
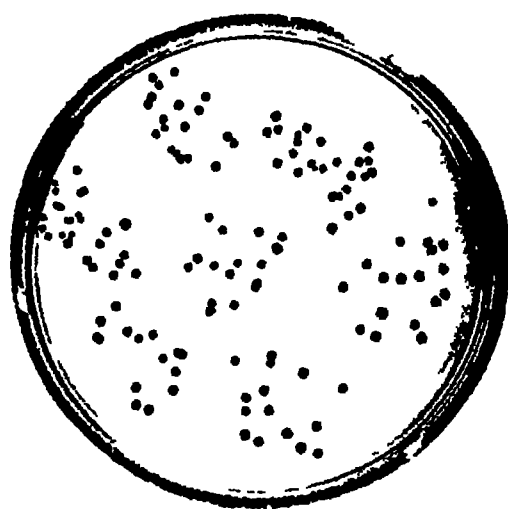
Haemin 5 µg / ml
Aerobic
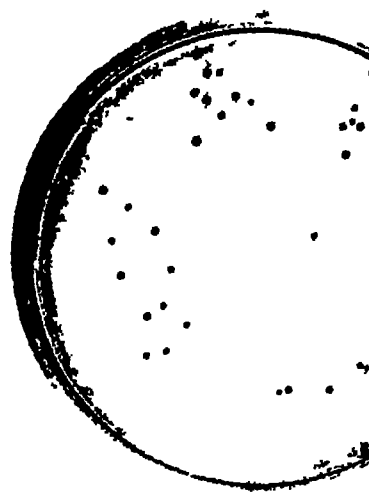
Haemin 1 µg /ml
Aerobic
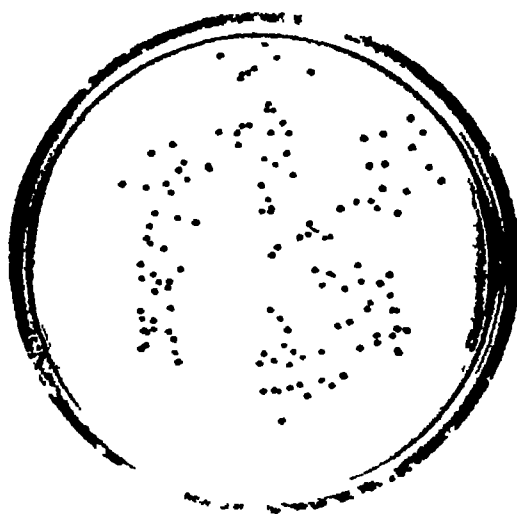
No Additions
Anaerobic

No Additions
Aerobic

FIG 1 —Effect of haemin and anaerobiosis, on growth of *Pasteurella pestis* on ag
Basal medium tryptic meat agar, with indicated additions of haemin All plate
the same inoculum (*ca* 140 cells) of a 24 hour culture of strain " Tjiwidej,"
incubated 40 hours at 37° C
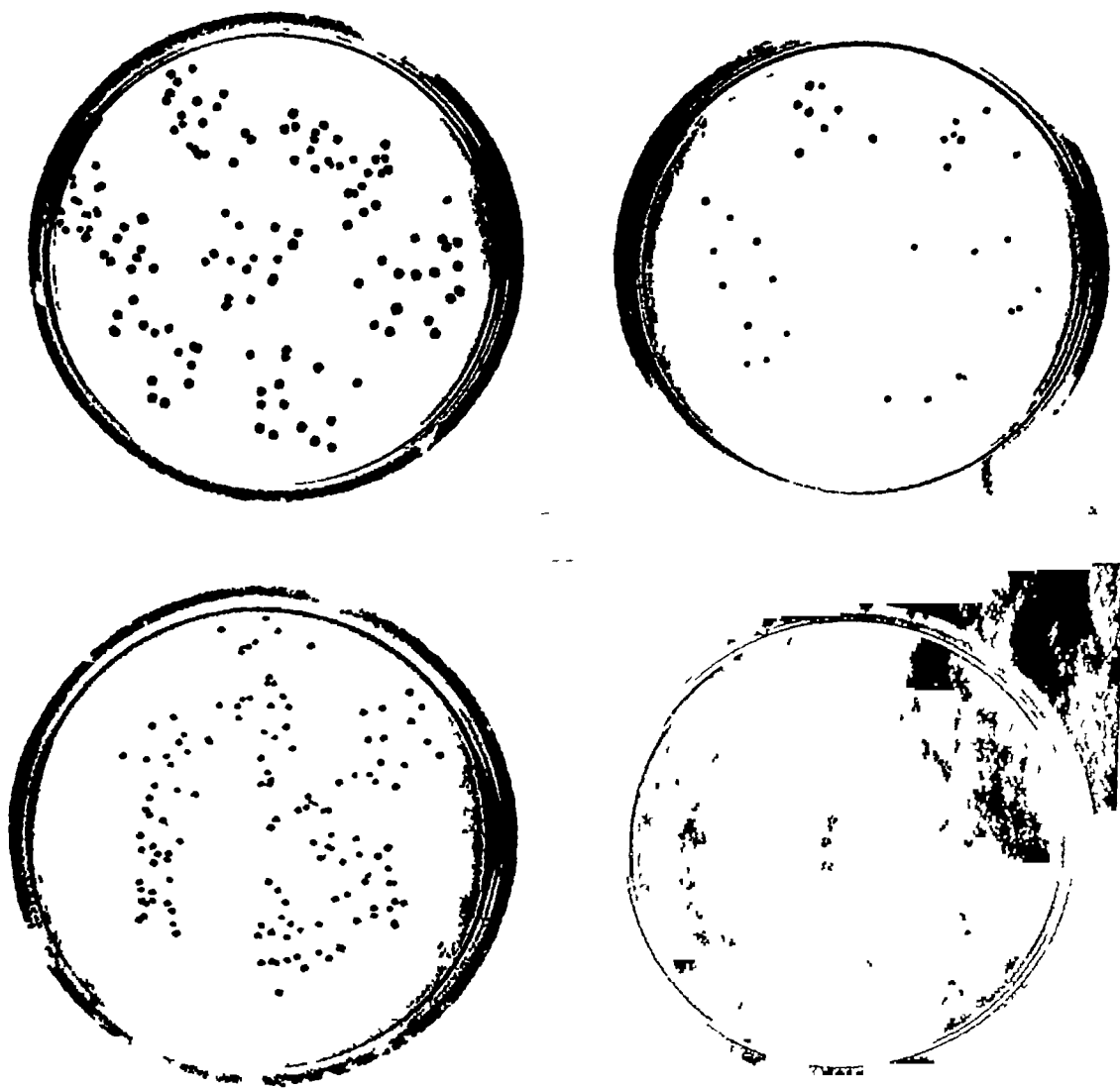
Fig 1 —Effect of haemin, and anaerobiosis on growth of *Pasteurella pestis* on agar plates
Basal medium tryptic meat agar, with indicated additions of haemin   All plates received
the same inoculum (*ca* 140 cells) of a 24 hour culture of strain ' Tjiwidej, and were
incubated 40 hours at 37° C

Herbert

TABLE V —*Effect of Various Substances on Aerobic Growth from Small I*

Additions to basal medium	Colonies developing in 40 hour		
	Exp 1	Exp 2	E
Nil	0	0	
5% pepsinized sheep s blood	242	259	
Haemin (10 µg /ml)	238	—	
Thiolacetic acid (0 1%)	220	—	
Na₂SO₃ (0 1%)	—	278	
Charcoal (B D H " blood charcoal ")	—	238	
Maize starch (0 2%)	0	—	
Glucose (0 5%)	—	—	

Basal medium tryptic meat agar   Thiolacetic acid and Na₂SO₃ sterilized by filtration, r
substances by autoclaving   All plates in each experiment seeded with 7 drops of the same sus
and incubated aerobically at 37°

The " haematin effect " is independent of the basal medium used, id
results being obtained with tryptic meat digest agar, Evans' peptone, and
hydrolysate
All of the five strains tested (3 highly virulent and 2 completely av
showed the " haematin effect "

Absence of haematin in ordinary bacteriological media

The above experiments show the effect of haematin to be maximal at 5
and detectable at 1 µg /ml , since no growth at all occurred on the basal
they presumably contain less than 1 µg haematin/ml   In the case of
digest agar this is unexpected, since the meat from which it is made is
haematin compounds   Most of the haematin is removed during the prepa
of the medium, with the coagulum formed when the digested meat is stea
pH 6 , even so, it seems surprising that small traces do not remain
The probable explanation was discovered when a batch of medium w
countered on which isolated colonies grew without added haemin   It was
that in its preparation the usual " dephosphating " procedure, warming
8 6 to precipitate calcium phosphate, and filtration, had accidentally been on
Another batch of medium was prepared, half of which was dephosphated
the remainder was not   Agar plates made from the dephosphated broth
no aerobic growth of *Past pestis* unless haemin was added   Plates made
the broth that had not been dephosphated gave submaximal growth w
addition of haemin, about 50 per cent of the expected number of colonies de
ing , this corresponds to the effect produced by adding about 2 µg haemi
to haemin-free media
A model experiment was carried out by adding haemin (10 µg /ml) t
water (containing *ca* 100 mg calcium/litre) adjusted to pH 8 6, then a
M/100 phosphate of the same pH, and centrifuging   Quantitative estima
showed that all the haematin had been carried down on the calcium phos
precipitate, no detectable amounts remaining in the supernatant
Pappenheimer and Shaskan (1944) removed traces of iron from simp
media by adding calcium salts and phosphate, all the inorganic iron
adsorbed on the calcium phosphate precipitate   Evidently the same techi

will be useful when it is desired to render complex media completely free from haematin

Effect of other substances on aerobic growth from small inocula

These results suggest that (*a*) *Past pestis* can synthesize haematin with difficulty or not at all, and (*b*) that haematin might be the prosthetic group of some enzyme which is necessary for aerobic growth The effect of KCN, which inhibits iron-porphyrin enzymes such as catalase, peroxidase and cytochrome oxidase, was therefore tested (Table IV) Anaerobically, KCN in concentrations up to M/250 had no effect, and M/50 a scarcely significant effect, whether haemin is present or absent Aerobically, no colonies developed unless haemin was present but if KCN was also present it abolished the effect of haematin completely at M/250 by 90 per cent at M/1000 and by 50 per cent at M/5000 (The actual concentrations present after 40 hours were probably less than these, owing to the volatility of HCN)

These results agree with the above hypothesis The position became more complicated, however, when other substances were tested in place of haemin It was then found (Table V) that sodium sulphite, thiolacetic acid and animal charcoal would promote the aerobic growth of isolated colonies in exactly the same way as peptic blood and haemin , starch and glucose, however, had no effect Charcoal and starch were tested because of their stimulating action on the growth of gonococci and meningococci (Glass and Kennett, 1939 , Mueller and Hinton, 1941)

It should be pointed out that haemin is effective in concentrations at least 100 times lower than the other active substances tested

Experiments on chemically defined media

The above results are open to various interpretations, and it was thought that a clearer insight into the factors concerned would be gained by studying the nutrition of *Past pestis*, and conducting experiments in a chemically defined medium Three workers (Rao, 1939 , Berkman, 1942 , Doudoroff, 1943) have already studied this problem, but their results are so conflicting that it was necessary to approach the matter afresh

It was found that none of the five strains available would grow on the simple media containing only two or three amino-acids described by Rao (1939) or Doudoroff (1943) The next medium tested was similar to that of Berkman (1942), containing 20 amino-acids, ammonia, salts and glucose With this medium, results were irregular Usually no growth occurred, but occasionally some flasks grew up after 3–5 days' incubation This effect was erratic, e g in duplicate flasks sometimes one would grow when the other did not, and usually growth only occurred when a heavy inoculum was used, e g 10^6 cells/ml It was concluded either that only a very small proportion of cells in any culture was capable of growth in this medium, or else that actual mutation or " training " was sometimes occurring

Table VI shows the effect of adding haemin, and mixtures of 17 other growth factors, to the amino-acid medium No growth occurred with the amino-acids alone, or with the 17 other growth factors, while good growth occurred with haemin 5μg /ml of haemin gave a maximum effect and 1μg /ml a detectable

TABLE VI —*Growth in Amino-acid Medium, Effect of Haemin and Other Growth Factors*

Additions to amino acid medium	Growth after incubation at 37° for—			
	24 hours	40 hours	48 hours	3 days
Nil	0	0	0	0
Haemin 1 µg /ml	0	Trace	±	+ +
,, 2 ,,	0	+	+ ±	+ +
,, 5 ,,	0	+ +	+ + +	+ + ±
,, 10 ,,	0	+ +	+ + +	+ + ±
Growth factors I	0	0	0	0
,, ,, I + II	0	0	0	±
,, I + haemin (10 µg /ml)	0	+ + +	+ + +	+ + ±
,, ,, I + II + haemin (10 µg /ml)	Trace	+ + +	+ + +	+ + ±

Ten ml complete amino acid medium with M/100 glucose salts and the indicated additions, inoculated with 1250 viable cells/ml Strain 337 Growth Factors Group I Nicotinamide, 5×10^{-6} M pyridoxin, pantothenic acid, riboflavine, 2×10^{-6} M , p aminobenzoic acid, thiamine, 10^{-6} M , biotin 10^{-3} g /ml Group II Glutamine, 2×10^{-1} M , choline, uracil, adenine, guanine, thymine, xanthine, inositol, 5×10^{-5} M , oleic acid, 2×10^{-5} M , pimelic acid, 5×10^{-6} M

effect—exactly the same concentrations as those found effective by the agar plate technique (Table III) When the 17 growth factors were added in addition to haemin, growth was slightly, but consistently, faster than with haemin alone Further experiments showed that no single growth factor alone had any noticeable effect, while various mixtures of them had slight accelerating effects which appeared to be additive

The conclusion was that *Past pestis* is capable of synthesizing all these growth factors, but will grow rather faster if they are supplied in the medium The effect of haemin was of quite a different order, since without it growth was always greatly delayed, and usually did not occur at all, particularly if small inocula were used The amino-acid medium with haemin consistently supported growth with inocula as small as 10 cells/ml Strain 337 was still highly virulent for mice after 13 sub-cultures in this medium

All of the five strains tested grew on this medium The amino-acid requirements of one of them, virulent Strain 337, were studied in some detail The effect was first tested of omitting each of the amino-acids singly from the complete amino-acids-haemin medium The results showed that the amino-acids fall into four groups

(1) *Omission from the medium has no effect*—glycine, alanine, norleucine, aspartic and glutamic acids, lysine, proline, hydroxyproline, tyrosine and tryptophane

(2) *Omission causes slight delay in growth*—serine methionine, arginine

(3) *Omission causes marked delay in growth*—threonine, histidine

(4) *Omission causes complete absence of growth*—valine, leucine, isoleucine, cystine and phenylalanine

From this experiment, which was repeated with identical results, most of the amino-acids appeared to be dispensable and only those of Group 4 to be " essential " However, a mixture of the five " essential " amino-acids with ammonia, glucose and haemin did not support growth, even when threonine, histidine and

TABLE VII —*Growth on Mixtures of Amino-acids*

Amino acid mixture	Growth after incubation at 37° for—			
	16 hours	24 hours	40 hours	48 hours
(1) Valine, leucine, isoleucine, cystine, phenyl-alanine	0	0	0	0
(2) As 1 + threonine and histidine	0	0	0	0
(3) As 2 + proline	0	0	0	6
(4) As 3 + glycine, alanine and glutamic acid	0	Trace	+	++
(5) As 4 + serine and methionine	0	±	++	+++
(6) Complete amino-acid medium	Trace	++	+++±	+++

Amino acids in same concentrations as previous experiments. All mixtures contained M/100 glucose, salts and haemin (5 µg /ml). Inoculum 10⁶ cells/ml. Strain 337

proline were also added (Table VII) Further addition of glycine, alanine and glutamic acid gave moderate growth, and with these plus serine and methionine the growth was fairly good, though still slower than on the complete amino-acid medium This may not be the simplest possible mixture adequate for growth, but it is evident that the minimum number of amino-acids required is not small

The other four strains were not investigated in detail All required some amino-acids, since they would not grow on an ammonia-salts-glucose medium, even when haematin and 17 other growth factors were added None required tryptophane, since growth occurred on an acid-hydrolysed casein medium (with glucose and haemin) known by microbiological tests to be tryptophane-free

These results show that for growth under the conditions employed, which are fairly aerobic, thin layers of liquid in Erlenmeyer flasks, *Past pestis* requires a number of amino-acids and haemin Table VIII shows the effects of haemin under aerobic and strictly anaerobic conditions It will be seen that anaerobically, growth is equally rapid whether haemin is present or absent, while aerobically no growth occurs without haemin Haemin can be replaced, however, by the reducing agent thiolacetic acid, in whose presence aerobic growth occurs without haemin as it does anaerobically

Comparison of these results with those of Tables III to V shows that the effects of haemin, reducing agents and anaerobiosis are essentially the same in a chemically defined liquid medium as they are on nutrient agar plates

TABLE VIII —*Effect of Haemin, Thiolacetic acid, and Anaerobiosis, on Growth in Amino-acid Medium*

Additions to amino acid medium	Aerobic			Anaerobic		
	24 hours	48 hours	3 days	24 hours	48 hours	3 days
Nil	0	0	0	0	++±	++±
Thiolacetic acid (M/100)	0	++±	+++	0	++±	++±
Haemin (5µg /ml)	Trace	+++	+++	0	++±	++±

Ten ml complete amino acid medium with M/100 glucose, salts, and indicated additions inoculated with 2000 cells/ml "Aerobic" in air/5 per cent CO₂, "Anaerobic" in hydrogen/5 per cent CO₂ in McIntosh and Fildes anaerobic jar Strain 337

Catalase

Bacterial catalase, like that of higher animals, is a haematin-protein (Herbert and Pinsent, 1948)   Bacteria unable to synthesize haematin are therefore unable to synthesize catalase unless haematin is supplied in the growth medium

All strains of *Past pestis* tested produced an active catalase when grown in the amino-acids-glucose medium under the following conditions

(*a*) Aerobic or anaerobic growth in the presence of haemin

(*b*) Anaerobic growth in the absence of haemin

(*c*) Aerobic growth without haemin in the presence of reducing agents, such as thiolacetic acid

(*d*) On the rare occasions when growth was observed, usually from large inocula, in the absence of either haemin or a reducing agent

In short, whenever the organisms grew at all, they produced catalase, whether haemin was supplied in the growth medium or not   The same was true of growth on agar media

Ability to synthesize catalase in the absence of added haematin shows that the organisms can themselves synthesize haematin   The bearing of this on the ' haematin effect " is discussed below

DISCUSSION

The nutrition of *Past pestis* has been studied by three previous workers, with rather discrepant results   Berkman (1942) found that four out of five old laboratory cultures grew in a medium containing 15 amino-acids and glucose, without added growth factors   Rao (1939) claimed that a medium consisting only of the three substances cystine, phenylalanine and proline was sufficient to support growth   Doudoroff (1943) found that five strains would grow in an ammonia-salts-glucose medium with cystine and phenyl-alanine added   None of these authors found haemin to be essential for growth, though Rao reported that it had an accelerating effect

None of the strains tested in this work would grow in media as simple as those of Rao or Doudoroff   Anaerobically they would all grow in a mixture of 20 amino-acids and glucose, but for aerobic growth haemin was also required, at least when small inocula were used   This medium supported growth from inocula as small as 10 cells per ml   For one virulent strain whose requirements were investigated in detail, at least five amino acids were needed

Some of these discrepancies may be due to strain differences, but they are probably mainly due to differences in size of inocula   Rao used very heavy inocula, 0 2 ml per 10 ml tube of a suspension of opacity 1 on Brown's Scale and stated that inocula of 7000 viable cells per tube would not grow   Doudoroff stated, " Initially large inocula had to be used, and visible turbidity was slow to appear, evidence pointing to a selection of the cells most capable of reproducing in this environment "   The larger the inoculum, the greater the chance of including rare " mutants " with minimal growth requirement, and in liquid medium the development of only one cell out of the whole inoculum will be recorded as positive growth   Dr G M Hills informs me that he has found some strains of *Past pestis* to be nutritionally more exacting when grown at 37° than at 28° this may partly account for the varied findings of different workers as far as

amino-acid requirements are concerned However, I have observed the "haematin effect' on solid media to occur equally at 37° and at 30°

It seems probable that this is the reason why, in my experiments, growth was occasionally observed from large inocula in liquid synthetic media without haemin, while previous workers, using even larger inocula, have missed the "haematin effect' altogether

From this point of view the use of solid media is more satisfactory, and allows the "haematin effect" to be particularly clearly demonstrated, since the actual number of organisms in the inoculum which develop may be counted The ideal technique, no doubt, would be the use of chemically defined solid media, however results so far obtained show fairly clearly that the effect of haematin is essentially the same in liquid synthetic media and complex solid media

A complete explanation of the "haematin effect is not yet possible One hypothesis considered was that hydrogen peroxide produced by the bacteria under aerobic but not anaerobic conditions, was inhibiting aerobic growth The possibility of hydrogen peroxide originating from the growth medium might apply to tryptic digest agar, but presumably not to amino-acid media The effect of haematin might then be ascribed to its utilization by the organism for the synthesis of catalase, since HCN is a strong inhibitor of catalase, this could account for its reversal of the effect of haematin Animal charcoal, which can be substituted for haematin, catalytically decomposes hydrogen peroxide The effect of reducing substances such as sulphite or thiolacetate could be ascribed either to direct chemical reaction with hydrogen peroxide, or to their reaction with oxygen in the medium producing effectively anaerobic conditions, so that hydrogen peroxide is not formed

This explanation seems to be controverted by the fact that *Past pestis* can synthesize catalase in the absence of added haematin This objection may be only apparent Measurements of the catalase content of bacteria at different stages of the growth cycle (Pinsent, 1949) show that it is low in the early stages of growth and often does not reach its maximum until after growth has ceased In the development of isolated single bacteria it is probably the first few cell-divisions that are crucial, and it may well be that at this stage the cell has insufficient catalase for its requirements under aerobic conditions

A simpler, if vaguer, hypothesis is that growth depends on the oxidation-reduction potential of the medium, and can only take place if the E_h is below a certain level This could explain most of the observed phenomena, but it is difficult in this way to account for the effect of added haematin for it seems unlikely that, in a complex medium, haematin concentrations of a few μg /ml could directly affect the E_h

However, until more facts are known, it will remain easier to devise alternative hypotheses than to confirm or disprove them

SUMMARY

Heavy inocula of *Pasteurella pestis* grow aerobically on the surface of ordinary agar media, but widely separated single cells will not grow Isolated cells will grow, however, if whole blood or a peptic digest of blood are added to the agar

The factor in peptonized blood responsible for this effect was identified as haematin , the effect can be duplicated by the addition to plain agar of pure

recrystallized haemin, which has a maximum effect at a concentration of 5 μg /ml and a detectable effect at 1 μg /ml   The effect of haemin is abolished by the simultaneous addition of M/1000 KCN

This action of haemin is not a simple growth-factor effect, for under anaerobic conditions isolated cells will grow on plain agar without haemin, and growth is not inhibited by KCN   Also, isolated cells will grow aerobically in the absence of haemin if blood charcoal or reducing substances such as sodium sulphite or thiolacetate are added to the agar, though much higher concentrations of these are needed than of haemin

The nutritional requirements of *Past pestis* have been studied in chemically defined media   This organism requires none of the ordinary growth-factors, and grows anaerobically in a medium containing only synthetic amino-acids, salts and glucose   A preliminary study of the amino-acid requirements has been made

For aerobic growth from small inocula in amino-acid media, haemin or a reducing agent such as thiolacetate must be added, confirming the results obtained with complex agar media

As a preliminary hypothesis it is suggested that hydrogen peroxide is produced under aerobic conditions, and inhibits growth   Aerobic growth is made possible by adding reducing substances, which may act either by direct chemical reaction with hydrogen peroxide, or by rendering growth-conditions effectively anaerobic so that no peroxide is produced   Added haemin is assumed to act by promoting the synthesis of bacterial catalase

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SEROLOGICAL STUDIES WITH HERPES SIMPLEX VIRUS

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THE detection and measurement of antibody to herpes simplex virus in the serum of experimental animals or man has generally been made by neutralization tests   In these tests mixtures of serum and active virus have been injected into rabbits by the intracerebral route (Zinsser and Tang, 1929) or intradermally (Andrewes and Carmichael, 1930) or intracerebrally into mice (Weyer 1932, Hudson, Cook and Adair, 1936)   In 1939 Burnet and Lush carried out neutralization tests on the chorio-allantois of developing chick embryos   These methods give satisfactory results, but are expensive in both time and material   Bedson and Bland (1929) showed that herpetic antibody could be detected in the serum of immunized guinea-pigs by the complement-fixation test, using extracts of herpes-infected guinea-pig pads as antigen , by this technique antibody in human sera was demonstrated by Brain (1932), who found that approximately 45 per cent of sera from unselected hospital patients gave a positive reaction   Other workers have apparently not been able to obtain consistently satisfactory results by this *in vitro* method (Myers and Chapman, 1937), and in two recent American works on virus diseases only the neutralization test is recommended for the detection of antibody to herpes simplex virus in human sera (Scott, 1948 , Scott, Coriell and Blank, 1949)

In the course of the present work the results of Bedson and Bland (1929) were readily confirmed in that antibody to herpes simplex virus in the serum of immunized animals could readily be measured by their complement-fixation technique   As we had available several strains of herpes virus which had been adapted to the chorio-allantois, the tissues and fluids from infected chick embryos have been examined for their suitability as antigens in the complement-fixation test   As shown by Anderson (1940), egg adapted strains of herpes virus produce generalized infection of chick embryos , after inoculation of such strains into the yolk sac or amniotic cavity, virus is present in high concentration in the amniotic fluid several days later (Nagler, 1946 , Beveridge and Burnet, 1946)   Extracts of herpes-infected chorio-allantoic membranes, amniotic and allantoic fluids have been successfully used in the present work as antigens in complement-fixation tests

MATERIAL AND METHODS

Virus strains

Four strains of herpes virus have been studied   Strain " N " was isolated directly in chick embryos by the inoculation of the chorio-allantois with vesicle fluid from a case of a generalized infection with herpes simplex virus (Kipping and Downie, 1948)   The other three strains, " H," " McC " and " P," have been

isolated within the last 12 months from members of the staff who suffer from recurrent attacks of labial herpes   In each case the virus was isolated without difficulty by the inoculation of the chorio-allantois of developing eggs with diluted vesicle fluid

Preparation of the antigens

In the inoculation of chick embryos by various routes the techniques described by Beveridge and Burnet (1946) have been followed

Chorio-allantoic membrane extracts

Twelve-day-old embryos were inoculated on the chorio-allantois and the membranes were removed after two days' further incubation   They were ground up in a tube with powdered glass by means of a glass rod and then extracted with saline containing 20 per cent nutrient broth, approximately 0 5 ml being added for each membrane   After being kept, with occasional shaking, for 2 hours at room temperature, the suspension was centrifuged at low speed until the supernatant fluid was clear (5–10 mm)   The supernatant was removed and used as the antigen

Extra-embryonic fluids

Allantoic and amniotic fluids were harvested within 24 hours after the death of the embryo following either yolk sac, amniotic or allantoic inoculation with a suspension in broth of herpes-infected chorio-allantoic membrane

Antisera

Immune human serum against three strains, " H," " McC " and " P," were obtained from the individuals who furnished the virus strains   Immune sera were prepared against each strain by immunization of guinea-pigs   To avoid the formation of antibody to chick protein herpes-infected guinea-pig pads were used as the source of inoculum in each case   The infected pads were ground finely and approximately 5 ml of sterile nutrient broth were added   The suspensions were treated with sufficient penicillin, and in the case of strain " H " with streptomycin as well, so that subsequent cultures on agar and in broth showed no bacterial growth   Each preparation contained sufficient virus to produce a confluent growth of typical herpetic lesions on the chorio-allantois   The strain " N " had undergone 30 previous egg-to-egg passages on the chorio-allantois, strains " H," " McC " and " P " had undergone 15, 7 and 4 respectively   Guinea-pigs " H," " P " and " N " each received their first two inoculations intradermally into the plantar skin of both hind pads at an interval of two days   On the 4th day after the primary inoculation the soles of the hind feet were swollen and vesicles were developing along the needle tracks   On the 8th day " N " had developed paralysis of both hind limbs, from which it never completely recovered   The animals each received a subcutaneous inoculation of virus on the 31st day, and two further intraperitoneal inoculations on the 52nd and 59th days after the primary inoculation   A week later guinea-pig " N " appeared extremely excitable and was bled out from the heart   Animals " H " and " P " each received two further intraperitoneal injections on the 66th and 73rd days and were bled a week later   Guinea-pig " McC " received its first inoculation

intradermally into the plantar skin of both hind pads at the same time as the other animals As the feet showed signs of an early " take " after two days, it was not given a further inoculation at this time A week later there was paralysis of the hind limbs, but this had cleared up within a fortnight On the 37th day after the primary inoculation the animal was given a subcutaneous injection of virus Fourteen days later the right hind leg was badly inflamed and the animal was bled Immune rabbit serum had been prepared against strain " N " by immunization with active virus obtained from infected rabbit testicle (Kipping and Downie, 1948) Immediately before use all sera were inactivated by heating at 56° C for half an hour

Technique of Complement fixation Tests

The unit volume of reagents used was 0 2 ml Antigen dilution, complement and serum were mixed and kept in the refrigerator at 4° overnight (18 hours) Next morning the tubes were kept at room temperature for 30 minutes before adding 0 4 ml of sensitized sheep red cell suspension (equal volumes of 2 per cent red cell suspension and amboceptor diluted to contain 3 M H D) The tubes were kept in a water bath at 37° for 30 minutes and then at room temperature for 1–2 hours to allow red cells to settle before the readings were made

When extracts of chorio-allantoic membrane were used as antigen 2½ M H D of complement was used in the tests, but with amniotic or allantoic fluid antigens 2 M H D was used All serum and antigen control tubes received ½ dose less complement than the tests proper Tests with extra-embryonic fluids or chorio-allantoic membrane extracts from normal uninoculated eggs were set up with all experiments Serum from normal guinea-pigs or rabbits was similarly tested with immune sera from these species, and negative human serum from one of several non-herpetic subjects on the staff of the department was used to control the tests with human sera

RESULTS

Complement fixation with Antigens Prepared from Infected Chorio-Allantoic Membranes, Allantoic and Amniotic Fluids

The first table shows the results with chorio-allantoic antigen, falling dilutions of the antigen being tested with falling dilutions of immune human serum

TABLE I —*Titrations of Chorio-Allantoic Membrane Extract with Dilutions of Immune Human Serum*

Antigen dilutions	Dilutions of immune serum				Negative human serum		No serum
	1/2	1/4	1/8	1/16	1/2	1/4	
1/4	++++	++++	++++	+++	—	—	—
1/8	++±	+++	+++±	+++	—	—	—
1/16	++	++	+++±	++	—	—	—
1/32	+	+	+	+	—	—	—
1/64	—	—	—	—	—	—	—

++++ = Complete fixation
— = No fixation

+++ ⎫
++ ⎬ Indicate degrees of fixation less than complete
+ ⎭

Similar results were obtained using immune rabbit serum except that the rabbit serum had a higher antibody content than the human serum, as shown by the higher dilution of serum which gave positive reactions

Table II shows the results when human serum diluted 1/8 was used to test falling dilutions of allantoic and amniotic fluids

TABLE II —*Titration of Allantoic and Amniotic Fluids with Immune Human Serum diluted 1/8*

Antigens	Immune human serum 1/8						Negative human serum 1/8		No serum
	Dilutions of antigens								
	1/4	1/8	1/16	1/32	1/64	1/128	1/4	1/8	1/4
Amniotic fluid	++++	++++	++++	++++	+++	—	—	—	—
Allantoic fluid	++++	++++	++++	++±	—	—	—	—	—
Normal fluid	—	—	—	—	—	—	—	—	—

Table III shows the results when falling dilutions of immune guinea-pig sera ' McC ' and ' N ' were titrated against constant antigen—allantoic fluid diluted 1/8

TABLE III —*Titration of Immune Guinea-pig Sera with Constant Antigen (Allantoic Fluid diluted 1/8)*

Sera	Infected allantoic fluid 1/8						Norman allantoic fluid		No antigen
	Dilutions of guinea pig sera								
	1/8	1/16	1/32	1/64	1/128	1/256	1/8	1/16	1/8
" McC "	++++	++++	++++	++±	—	—	—	—	—
" N "	++++	++++	++++	++++	++	—	—	—	—
Normal	—	—	—	—	—	—	—	—	—

The constant dilution of antiserum or antigen used for titration of antigen and antiserum respectively as shown in Tables II and III were chosen, on the bases of preliminary tests of the kind shown in Table I, as those likely to give high titres

The Nature of the Antigen and its Stability to Heat and Storage

The activity of the three antigens—allantoic fluid, amniotic fluid and chorio-allantoic membrane extract—appears to be related not to the virus particle itself but to some " soluble " specific substance separable from the virus by centrifugation

To elicit this point the antigens were spun in an angle centrifuge at approximately 8000 r p m for one hour The upper half of the supernatant was carefully removed, the lower half was discarded, care being taken not to disturb the deposit The tube was well drained, and the deposit resuspended in saline containing 20 per cent nutrient broth The centrifugation was repeated, the supernatant fluid removed as before, and the deposit again resuspended to its original volume

The complement-fixing power of the original fluid, 1st supernatant, 2nd supernatant and resuspended deposit were tested against immune human or guinea-pig sera

Tests of the infectivity of the original fluid and 1st supernatant, determined by titration on the chorio-allantoic membranes of developing chick embryos, showed that the 1st supernatant contained less than 2 per cent of the virus present in the original fluid

Table IV shows the results of testing various fractions of allantoic fluid with immune human serum diluted 1/8

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TABLE IV.—*Titration of Various Fractions of Infected Allantoic Fluid with Immune Human Serum*

Antigens	Immune human serum (1/8) — Dilutions of antigens							Negative human serum (1/8)				No serum	
	Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	Undiluted	1/2	1/4	1/8	Undiluted	1/4
Original fluid	0	0	++++	++++	++++	+++	+	0	0	—	—	0	—
First supernatant	0	0	++++	++++	++++	+++	—	0	0	—	—	—	—
Second supernatant	—	—	—	—	—	0	0	—	—	0	0	—	0
Resuspended deposit	—	—	—	—	—	0	0	—	—	0	0	0	0

0 = Not tested

An essentially similar result was obtained when infected amniotic fluid and mixtures of amniotic and allantoic fluids were tested in the same way against a constant dilution of immune human serum

Examination of an antigen prepared from infected chorio-allantoic membrane gave the results shown in Table V   The infectivity of this antigen was not tested   However, titration of another membrane extract similarly prepared showed that approximately 98 per cent of the virus was deposited during the first centrifugation   It will be seen that the resuspended washed deposit showed more fixation of complement than similar deposits from amniotic and allantoic fluid antigens   This difference has been apparent in several experiments of this kind, and suggests that soluble antigen is more difficult to remove from the tissue particles deposited with the virus from extracts of infected chorio-allantois

TABLE V —*Titration of Various Fractions of Chorio-Allantoic Membrane Extract with Immune Human Serum (1/8)*

Antigens	Immune human serum 1/8					Negative human serum 1/8			No serum	
	Dilutions of antigen									
	1/2	1/4	1/8	1/16	1/32	1/2	1/4	1/8	1/2	1/4
Original fluid	0	++++	++++	+++	++	0	—	—	0	—
First supernatant	++++	++++	+++	++	—	—	—	0	—	0
Second supernatant	—	—	—	—	—	—	—	0	—	0
Resuspended deposit	+++	++	—	—	—	—	—	0	—	0

0 = Not tested

The effects of heating the " soluble " antigen at 56° for one hour and of boiling for 5 minutes are shown in Table VI   The antigen used was the first supernatant from the high speed centrifugation of infected allantoic fluid   It is apparent that the complement-fixing activity is greatly weakened by heating at 56° for 1 hour, but is not completely destroyed even by boiling for 5 minutes

TABLE VI —*Effect of Heat on the " Soluble " Antigen*

Antigens	Immune human serum 1/8						Negative human serum 1/8		No serum	
	Dilutions of antigen									
	1/2	1/4	1/8	1/16	1/32	1/64	1/2	1/8	1/2	1/8
Unheated	0	0	++++	++++	++++	—	0	—	0	—
Heated 56° 1 hour	++++	++++	+++	—	—	—	—	0	—	0
Boiled 5 min	++++	±	—	—	—	—	—	0	—	0

0 = Not tested.

An infected allantoic fluid was tested after storage for three months in a refrigerator at approximately 4°   Using the same immune human serum for test before and after storage there appeared to have been little, if any, fall in the

complement-fixing titre of the fluid during this time   The effect of treatment of the antigen with formalin has not been investigated, but it appears that infected chick embryo fluids when stored at low temperature, may be used for the titration of antibody in sera over a period of several months

A Serological Comparison of Four Recently Isolated Strains of Herpes Virus by Complement-Fixation Technique

There is very little evidence in the literature of immunological diversity among strains of herpes virus   Florman and Trader (1947) studied four strains of diverse origin   using immune sera prepared in rabbits they found that by neutralization tests in mice and on the chorio-allantois, three strains appeared to be immunologically identical, while the fourth was only partially neutralized by the sera prepared against the other three strains   Slavin and Gavett (1946) similarly found that one of three strains differed from the other two in neutralization tests in mice using immune rabbit sera

Sera from the individuals who provided three of the herpes strains available to us were used in tests of amniotic fluid antigens from all four strains   The fluids were used in a dilution of 1/4 in two instances and 1/8 in the other two the results are shown in Table VII

The three sera when tested against allantoic fluid antigens gave essentially the same results as those shown in Table VII   The results with the sera from immunized guinea-pigs are shown in Table VIII

Although two of the guinea-pig sera were very weak in complement-fixing antibody it is clear that these tests, like the tests with the human sera give no indication of strain specificity among the four viruses

It is perhaps of interest that the two weak guinea-pig antisera were obtained

TABLE VII —*Titration of Immune Human Sera " H,' " McC and P*

Antigens (diluted

Sera	H				McC			
	1/4	1/8	1/16	1/32	1 4	1/8	1 16	1 32
"H'	++++	++++	++++	++	++++	+++±	----	-
"McC"	++++	++++	++	-	+++±	++± ±	=	-
"P"	++++	++±+	++	+	++++	+±++	--	-
Negative serum	-	-	0	0	-	-	0	0

TABLE VIII —*Titration of Immune Guinea-pig Sera   H   McC,'*

Antigens (diluted

Sera	"H' (allantoic fluid 1/8)						McC (amniotic fluid 1 8)						1/4
	1/4	1/8	1/16	1/32	1/64	1/80	1/4	1/8	1/16	1/32	1/64	1/80	
"H"	+	+	++±	-	-	-	-	+	+±	+	-	-	-
"P"	±	-	-	-	-	-	±	-	-	-	-	-	±
"McC"	0	0	++++	++++	++++	-	0	0	++++	++++	++++	-	0
"N"	0	0	++++	++++	++++	+	0	0	++++	++++	++++	±++±	0
Negative	-	-	0	0	0	0	-	-	0	0	0	0	-

from animals that had received a full course of inoculations with living virus comprising two intradermal, one subcutaneous and four intraperitoneal injections over a period of approximately ten weeks   Neither of those two animals showed evidence of paralysis   The two animals which gave good complement fixing antisera, on the other hand, had a smaller number of immunizing injections, but both showed paralysis of the hind limbs following the pad inoculations

The Complement fixation Test for the Detection of Antibody in Human Sera

Various workers have shown that neutralizing antibody for herpes virus is present in a large proportion of adult sera, and that in general those showing such antibody have a history of recurrent herpetic infection   Only Brain (1932) appears to have used the complement-fixation technique in such work   He found that of 52 unselected sera from London hospital patients 23 gave complement fixation with herpetic antigens   We have tested 40 sera which had been sent to the Liverpool City Laboratory for Wassermann test   Of these sera only seven were negative in the complement-fixation test with a mixed allantoic and amniotic herpetic antigen   These sera were tested in a dilution of 1/4 and greater   Of the 33 which gave a positive reaction all but one (which was positive 1/32) were positive in dilutions up to 1/64, the titres being 1/64 or 1/128   The results bore no relation to the results of the W R   The sera could hardly be regarded as representative specimens from the general population, but the percentage of positives (82 5), although higher than that recorded by Brain, is not higher than the figure given by Burnet and Lush (1939) for neutralizing antibody in the sera of hospital patients in Melbourne   The results in this series also show the same all or nothing kind of result in that sera were either completely negative or had high complement fixing titres

against Antigens (Amniotic Fluids) "H," "P," "McC" and "N"

1/4 or 1/8)

"P" (Serum dilutions)				"N"				Normal amniotic fluid		No antigen
1/4	1/8	1/16	1/32	1/4	1/8	1/16	1/32	1/4	1/8	1/4
++++	++++	++++	++	++++	++++	++++	++	−	−	−
++++	++++	±	−	++++	++++	±	−	−	−	−
++++	++++	+	−	++++	++++	+	−	−	−	−
−	−	0	0	−	−	0	0	−	−	−

P" and "N" against Antigens "H," "McC," "P" and "N"

or 1/8)

P ' (allantoic fluid 1/4) (Serum dilutions)				"N " (amniotic fluid 1/8)						Normal allantoic fluid		Normal amniotic fluid		No antigen
1/15	1/32	1/64	1/80	1/4	1/8	1/16	1/32	1/64	1/80	1/4	1/8	1/4	1/8	1/4
+	±	−	−	−	+	+	±	−	−	−	−	−	−	−
−	=	−	−	±	−	−	−	−	−	−	−	−	−	−
++++	++++	++++	±	0	0	++++	++++	++±	−	−	−	−	−	−
++++	++++	++++	++±	0	0	++++	++++	+++	−	0	−	−	−	−
0	0	0	0	−	−	0	0	0	−	−	−	−	−	−

DISCUSSION

These experiments have shown that satisfactory herpetic antigens for complement fixation tests with immune sera from man or animals can be obtained from the amniotic or allantoic fluids of chick embryos infected with the virus of herpes simplex by various routes The strains of virus used yielded satisfactory antigens after a few passages on the chorio-allantois of chick embryos, by which time it was found that generalized infection of the embryos regularly following inoculation by the yolk sac or amniotic routes The antigen in allantoic and amniotic fluids is on the whole more satisfactory than that prepared from infected chorio-allantoic membranes as the fluids are almost devoid of anti-complementary properties The complement-fixing antigen may be obtained relatively free from virus and is relatively heat-labile, being greatly weakened by heating at 56° for one hour Fluids after boiling may retain slight complement fixing properties with immune sera suggesting that there may be a more stable component of the antigen in the experiments described, however such heat-stable antigen was apparently of little importance in the reactions obtained with unheated fluid

The experiments recorded afford no proof that the complement-fixing antibody in immune sera is the same as that concerned with neutralization of the virus However, ten sera were tested both by the complement fixation technique and by neutralization of herpes virus on the chorio-allantois there was complete correlation of the results obtained by the two methods Of 13 individuals whose history as regards herpetic infection was known the sera from all those and only from those with such a history gave a positive complement-fixation test Further, the proportion of positives found by complement-fixation on sera sent for routine Wassermann tests was similar to that recorded by neutralization tests in the hands of other workers, and the results on individual sera were similarly either completely negative or strongly positive No weak positive or doubtful reactions were encountered

SUMMARY

Complement-fixing antigen has been demonstrated in suspensions of chorio-allantoic membrane 2 days after inoculation with herpes simplex virus and in extra-embryonic fluids within 24 hours after the death of the embryo following either yolk sac, allantoic or amniotic inoculation Sera from immunized guinea-pigs and rabbits and sera from persons liable to recurrent herpetic infection served for the demonstration of the antigen

The activity of the complement-fixing antigen is chiefly due to a soluble specific substance separable from the virus by high-speed centrifugation

The activity of the " soluble ' antigen is largely destroyed by heat at 56° C for 1 hour , a slight degree of activity is still retained after boiling for 5 minutes The antigen can be stored with little loss at 4° for 3 months

Using the complement-fixation technique, no antigenic differences could be demonstrated among 4 recently isolated strains of herpes virus

The complement-fixation technique affords a sensitive method for detecting herpetic antibody in human sera, and is more economical of time and materials than neutralization tests

I am indebted to Professor A W Downie for encouragement and advice during the course of this work

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CIRCULATING FREE AMINO-ACIDS IN HYPOGLYCAEMIA

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In a previous communication (Mayer-Gross and Walker 1949) it was shown that the intravenous injection of 20 g of L glutamic, aminoacetic or para-amino-benzoic acid to subjects in hypoglycaemic coma could result in the restoration of consciousness and that while some rise in the blood glucose occurred in such cases, it was insufficient to account for the return of consciousness

In search of an explanation of this effect of the parenteral introduction of excessive amino-acids into the blood, it seemed of interest to consider the influence of hypoglycaemia on the normally circulating free amino-acids It had long been known that the free amino-acids in the blood are diminished in hypoglycaemia (Luck Morrison and Wilbur, 1928 Daniels and Luck, 1931, Harris, Blalock and Horwitz, 1938, Harris and Harris, 1947), and that the subsequent administration of glucose results in a further decrease in the circulating amino-acids (Luck Davis and Van Winkle 1935, Harris *et al*, 1938) Davis and Van Winkle (1934) and Crisman, Hanvey and Luck (1940) have ascribed this to the effect of adrenalin secreted in response to the injected insulin, because they found that the decrease in blood amino-acid does not occur when insulin is injected into the adrenalec-tomized animal, and that the injection of adrenalin itself to the intact animal produced hypoaminoacidemia If adrenalin is the agent which causes the diminution of amino-acids one would expect to find a definite relation between the amino-acid and blood glucose levels indicating the sympathomimetic action of adrenalin Moreover, the amino-acid content in subjects exhibiting greater restlessness and less depth of coma should differ from that in subjects who progressively go into a quiet and deep coma

There are, however, other possible explanations of the amino-acid decrease in hypoglycaemia besides adrenalinaemia Daniels and Luck (1931) have dis-cussed the hypothesis that insulin hypoaminoacidaemia may be due to an in-creased rate of glycogenesis from amino-acids, the hypoglycaemic state providing a stimulus to glycogenesis They also thought of a more direct influence of insulin on protein metabolism That this exists has been confirmed by more recent work Bouckaert and his collaborators (Laquet, de Nayer and Bouc-kaert, 1934, Bouckaert and de Duve, 1947) and Mirsky (1938) have shown that insulin exerts an inhibitory effect on proteolysis in the peripheral tissues as well as that it promotes or inhibits deamination in the liver, the latter being depen-dent on the blood glucose level

Some of these observations have a bearing on our special problem, and proved accessible to study on clinical material In the first part of the present paper serial experiments are reported demonstrating a quantitative relationship between plasma amino-acid and blood glucose levels in hypoglycaemia In the second place, the effect of adrenalinaemia on the amino-acid level is examined, using

clinical observations as a test without special regard to the blood glucose level Finally, an attempt is described to test hepatic deamination using galactose as an indicator of the speed of glycogenesis in the presence and absence of excessive amino-acid in the circulation

TECHNICAL

The subjects of the experiments were, as previously, patients undergoing Sakel's Insulin Coma Treatment and the same standard of coma was adopted An injectable preparation of L glutamic acid was made as described before (Mayer-Gross and Walker, 1949), and blood glucose determinations were made by the method of King, Haselwood and Delory (1937) Plasma amino-acid nitrogen determinations were made by the method of Folin as modified by Danielson, Sahyun and Frame, Russell and Wilhelmi (Hawk, Oser and Summerson 1947) Blood galactose was estimated by the same technique as blood glucose, with the exception that the isotonic sodium sulphate solution contained a fine suspension of washed yeast, and that the blood was allowed to remain in contact with this for ten minutes before precipitation of the proteins took place Under these conditions the blood glucose was completely fermented while the galactose was not affected By the use of standard solutions of galactose it was possible to compile a table of relationship between N/200 sodium thiosulphate solution and galactose

EXPERIMENTS AND RESULTS
Plasma Amino-acid and Blood Glucose

It was desirable to obtain comprehensive data of the concentrations of plasma amino-acid nitrogen, blood glucose and of the subject's state of consciousness over the whole period of " Insulin Treatment, ' from the point at which insulin was first given to that at which consciousness was fully restored after either a sucrose feed alone or combined with an intravenous injection of glucose

A series of 22 experiments was carried out in which simultaneous estimations of plasma amino-acid nitrogen and blood glucose were made before the injection of insulin and at hourly intervals thereafter until the point of onset of coma Observations were made at this point and 30 minutes later, immediately before the administration of 200 g of sucrose by a stomach-tube, furthermore at the point at which consciousness was restored and after a further 20 minutes If consciousness was not restored within 30 minutes of the administration of the sucrose the blood glucose and the plasma amino-acid nitrogen were estimated before the intravenous injection of 33 per cent (W/V) glucose solution The quantity injected varied from one case to another, but averaged 80 ml (25 g) After a further period of 15 minutes the observations on blood and plasma were repeated

The results of these experiments are set out in Table I, in which Columns 6 and 7 are alternative to Columns 8 and 9, depending upon whether or not it was necessary to give an intravenous injection of glucose in order to restore consciousness In 19 of the 22 cases it will be observed that up to the point of the onset of coma the concentration of plasma amino-acid nitrogen moves in the same direction as that of the blood glucose and the degrees of consciousness, and that it was less at the end of coma than at the start in 12 of the 22 cases Columns

TABLE I—*Concentration in mg/100 ml of Plasma Amino-acid Nitrogen (AAN₂) and Blood Glucose (Gl) before the Injection of Insulin and at Hourly Intervals Thereafter Until the Point of Onset of Coma, after 30 Minutes of Coma and (Col 6 and 7) at the Moment of Restoration of Consciousness after a Tube Feed of 200 g of Sucrose and 20 Minutes Later or (Col 7 and 8), if Consciousness had not been Restored, 30 Minutes after the Feed of Sucrose and 15 Minutes after an Intravenous Injection of Glucose*

Patient	1 Before Insulin		2 1 hour		3 2 hours		4 Coma onset		5 30 min coma		6 Awake		7 20 min later		8 60 min coma		9 15 min after IV glucose	
	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl

6, 7, 8 and 9 show that the administration of carbohydrate either in the form of sucrose by mouth alone or together with an intravenous injection of glucose frequently resulted in a further decrease in the plasma amino-acid nitrogen Examination of the figures in these columns, however, shows that although some of the amino-acid figures fall, others rise When the corresponding blood glucose level is less than approximately 80 mg /100 ml the plasma amino-acid continues to fall, and over that level the amino-acid concentration commences to rise

If such a critical level of blood glucose exists below which hypoaminoacidemia takes place, this had to be demonstrated in an experimental series with more critical observations, in which the blood glucose level during coma was varied by giving small amounts of intravenous glucose In eight patients the first couple of blood specimens were taken two hours after the injection of sufficient insulin to produce a state of hypoglycaemic coma, and thereafter at four 15-minute intervals after the intravenous injection of 5 g of glucose, and at two 15-minute intervals after the subsequent injection of 30 g glucose The results are set out in Table II In one case, Wy, Column 3, an increase in the plasma amino-acid nitrogen was noted at a blood glucose level of 12 mg /100 ml, and in five observations it remained constant despite a considerable degree of hypoglycaemia All the remaining observations, 42 in number, were in agreement with the principle noticed in the previous experiment That the blood glucose levels in Column 7 are in some cases higher than those in Column 6 is explained by the fact that after the injection of 30 g of glucose a quantity of sucrose dilution was given as a drink for the patient's benefit

The figures from Columns 6, 7, 8 and 9 of Table I and from Columns 2, 3, 4, 5, 6 and 7 of Table II are combined in the scatter diagram, Fig 1 which demonstrates the manner in which the rise and fall of the plasma amino-acid nitrogen is related to the level of the blood glucose, and in particular to the level of 80 mg / 100 ml

Plasma Amino-acid and Behaviour in Coma

There are marked individual variations of the subject's behaviour in hypoglycaemic coma Some reach the stage quietly and remain so while the unconsciousness deepens progressively during the 30 minutes before interruption In others the depth of coma fluctuates first and only later progressive deepening takes place Others again show much restlessness, myoclonic twitchings and

TABLE II —*Concentration in mg /100 ml of Plasma Amino-acid Nitrogen* (AAN_2) *and of Glucose* (Gl) *in Hypoglycaemic Patients Before and After the Successive Intravenous Inj of 5 and 30 g of Glucose*

Patient	1 2 hours after insulin		2 15 min after 5 g glucose		3 30 min after 5 g		4 45 min after 5 g		5 60 min after 5 g		6 15 min after 30 g		7 30 min 30 g
	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2	Gl	AAN_2
Tc	5 4	19	4 6	48	4 6	25	4 5	21	4 0	23	5 3	120	7 5
Col	4 4	23	5 5	92	4 4	25	4 4	23	4 3	16	4 9	87	5 1
Tw	5 1	28	5 0	35	5 0	28	4 9	28	3 6	23	4 9	90	6 9
Wy	5 9	12	4 8	14	6 2	12	5 6	12	5 6	9	6 2	93	7 1
McN	5 5	12	4 1	23	3 8	19	3 2	12	2 9	12	5 7	132	6 0
Fl	4 4	14	3 7	21	3 6	21	3 4	14	3 5	12	4 3	111	4 9
Th	5 3	9	3 9	14	3 8	9	3 6	7	3 0	9	5 4	118	6 1
Co	4 2	21	4 0	48	3 9	35	3 8	25	3 7	14	4 9	109	5 4
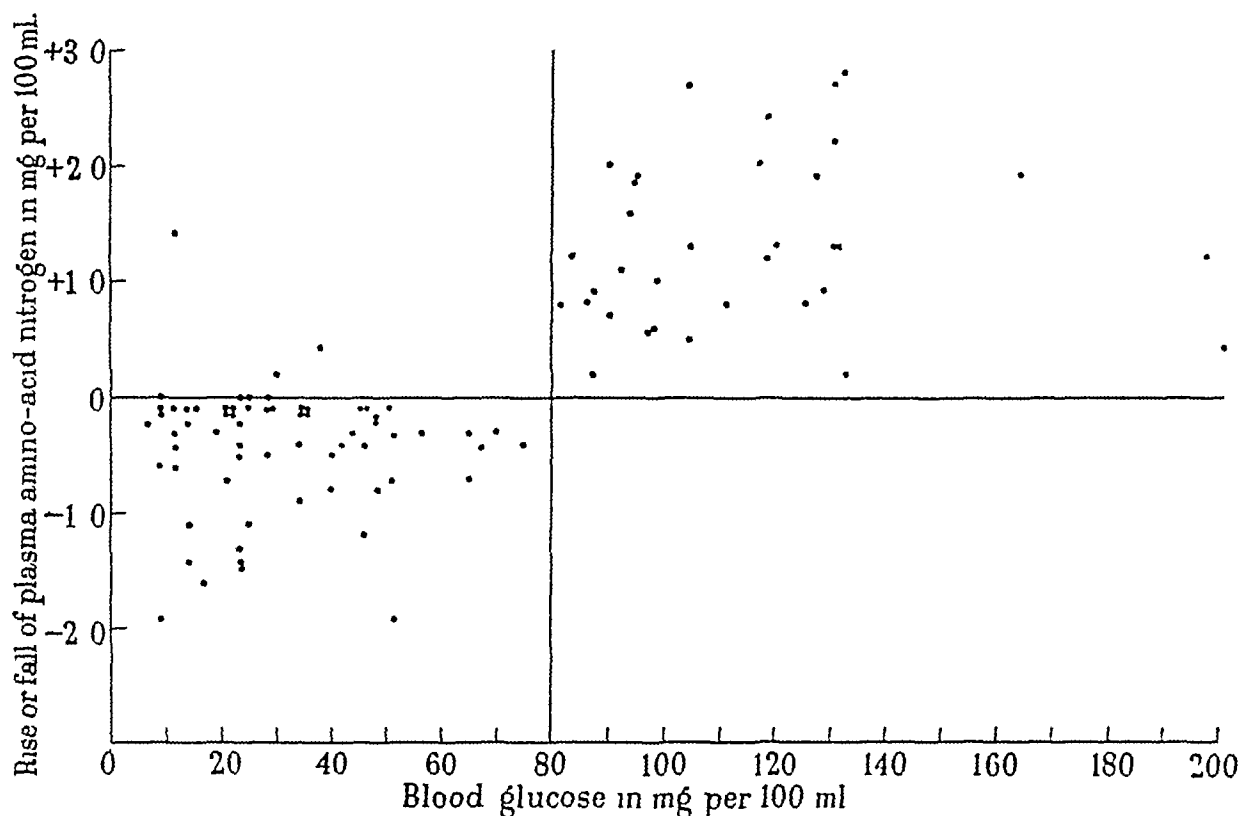
FIG 1 —Showing the rise or fall in plasma amino acid nitrogen after the injection of glucose to hypoglycaemic subjects plotted against the resultant blood glucose concentration

extrapyramidal movements   The fluctuations in depth are generally believed to be caused by the sympathomimetic effect of adrenalin counteracting the insulin   Restlessness increases the output of adrenalin into the circulation

Both phenomena can be enhanced by sensory stimulation—for instance by needle-pricks in the withdrawal of blood   If adrenaemia is, as was suggested, responsible for the lowering of the amino acid level, different values should be found parallel to the variations in the subject's behaviour in coma

In a series of fifteen experiments estimations of the plasma amino-acid nitrogen were made approximately 10 minutes before the patient became comatose, at the time at which coma commenced and at intervals of 10, 20 and 30 minutes thereafter   Notes regarding the patients' state of consciousness and behaviour were made at the same times   The results are summarized in Table III and show that in 13 of the 15 cases there is an average fall of 0 9 mg /100 ml in plasma amino-acid nitrogen at the time of the onset of coma   This fall is not always maintained, and is in several cases followed by a rise   In several cases where the coma was quiet, i e not complicated by variations in depth or by restlessness, there is a progressive fall, but in other similar cases this is not so   On the other hand, the first case, although complicated by great fluctuations in the depth of coma, shows a progressive fall in the concentration of plasma amino-acid nitrogen Three cases where excessive restlessness characterized the course of the coma, Numbers 2, 4 and 8, show a general tendency for the plasma amino-acid nitrogen to rise

TABLE III —*Concentration of Plasma Amino-acid Nitrogen in mg /100 ml 10 Minutes Before the Onset of Hypoglycaemic Coma, at the Moment of the Onset of Coma and at Intervals of 10, 20 and 30 Minutes Thereafter*

Number	10 mm before coma	Coma onset	10 mm coma	20 mm coma	30 mm coma
1	5 5	4 8	4 4F	4 2F	3 9
2	4 6	3 0R	3 3R	3 6	3 9
3	6 1	4 6R	3 9	5 1	4 7
4	3 5	4 6R	5 0R	4 8	5 4
5	4 5	3 8F	4 8F	5 0F	4 1
6	5 7	5 0	5 7F	5 0	4 4
7	5 5	4 7	4 8F	5 6F	6 1
8	5 0R	5 4R	5 5RF	5 5RF	5 7
9	5 7	5 5	4 6R	5 0	5 4
10	4 8	3 8	3 9	5 2	4 8
11	4 7	4 6	4 4	4 2	4 2
12	4 7	4 6	4 6	4 4	4 3
13	4 5	3 6	3 6	4 5	5 0
14	5 9	3 6	5 6	5 1	4 5
15	5 6	4 4F	4 5	4 0	5 2

F = Fluctuating coma , R = considerable restlessness

This series of experiments failed to show any direct relationship between the plasma amino-acid nitrogen and the behaviour of the subjects in hypoglycaemic coma The theory that the decrease in circulating amino-acids in hypoglycaemia is due to the action of adrenalin was not confirmed, since in those restless cases in which the output of adrenalin might be expected to be high the fall in the plasma amino-acid nitrogen was often the least marked

Influence of Excessive Amino-acids on Glycogenesis

Since the most probable reason for the withdrawal of free amino-acids from the circulation in hypoglycaemia is their deamination in the liver, and in view of the fact that this appeared to be reversed by the elevation of the blood glucose above 80 mg /100 ml , it appeared to be worth while to examine a complementary phenomenon, 1 e the effect of an abnormal amount of circulating amino-acid upon the rate of formation of glycogen in the presence of an excess of insulin For this purpose the rate of disappearance of galactose was observed when injected alone and in the presence of an excess of L-glutamic acid Galactose is converted into glycogen relatively slowly, and cannot be directly utilized in the tissues (Peters and Van Slyke, 1946)

Approximately two hours after the injection of sufficient insulin to produce hypoglycaemic coma, nine patients received intravenously 20 g of L-glutamic acid as well as 25 g of galactose After these injections parallel observations were made of blood glucose, blood galactose and plasma amino-acid nitrogen at intervals of 15, 30, 45, 60 and 90 minutes On a subsequent day a control experiment was carried out on the same subjects, receiving the same dose of insulin, who had been given only 25 g of galactose Blood glucose and galactose were estimated at the same intervals

The results are set out in Table IV, those of the experiments with glutamic acid in Columns 1–7 and those of the controls with galactose alone in Columns 8–14   When L-glutamic acid was injected the galactose concentration was greater and its persistence in the blood longer than in the control experiment   As an expression of concentration and persistence the " Galactose Index " is given for the experiments and controls in Columns 7 and 14 respectively   The figure was arrived at from the formula

$$\frac{\text{Sum of galactose concentrations in mg /100 ml} \times \text{time of persistence in minutes}}{100}$$

Its ratio between the experimental and control values is never less than 2   1, and in 6 of the 9 cases is greater than 5   1

Columns 1–6 of Table IV also show a reasonably constant relationship between the blood level of amino-acid nitrogen and the presence of circulating galactose   With the exception of the second case, CZ , galactose is not absent from the circulation while the concentration of plasma amino-acid nitrogen is substantially above that existing before the injection of L-glutamic acid and galactose   In Cases 1, 6, 7, 8 and 9 circulating galactose persists throughout the period of the experiment, and in each case the amino-acid nitrogen remained above the level originally observed   Similarly the glucose values were higher in the presence of injected glutamic acid than in its absence

If the glucose values from Columns 2–6 (experiments) and 9–13 (controls) are added up in each patient, one finds that in 6 of the cases the ratio is approximately 2   1 and in one case nearly 5   1 , in the remaining two cases the figures are approximately equal   Thus, in 7 of the 9 cases the quantity of circulating glucose under the experimental conditions was double that under those of the controls

DISCUSSION

If hypoglycaemia induced by an excess of insulin is regarded as a state of emergency, during which the homeostatically so very important level of blood glucose is artificially lowered, one finds that the different organs of the human body respond in a set order to this emergency   In earlier work we were able to show that, for instance, the cerebral cortex only becomes unresponsive, producing the state defined as coma, when the blood glucose was decreased to a level of 10 to 15 mg /100 ml (Mayer-Gross and Walker, 1945)   Depression of the function of the taste buds controlling the taste for sweetness already takes place at a blood glucose level of 50 mg /100 ml (Mayer-Gross and Walker, 1946)   Below this level our subjects selected from a number of drinks a concentrated sucrose solution, which they rejected because of its excessive sweetness above this level

The result of the first series of our present experiments suggests another critical blood glucose level   while plasma amino-acid decreases progressively under the influence of insulin as long as the blood glucose remains below 80 mg / 100 ml , this process is stopped and reversed when it rises above this level   Because of the further fall of amino-acid after the oral intake of sucrose and after waking up from coma, glucose and amino-acid levels seemed to be unconnected , in fact the mechanisms are linked up closely

If one tries to explain this finding, one has to consider the three relations

TABLE IV —Concentration in mg/100 ml of Plasma Amino-acid Nitrogen (AAN_2), Blood Galactose (Gal) and Blood Glucose (Gl) Before and After the Intravenous Injection of 25 g of Galactose and 20 g Glutamic Acid to Hypoglycaemic Patients (Experiments) and of Blood Glucose and Galactose After the Intravenous Injection of 25 g of Galactose Alone to the Same Subjects (Controls)

Experiments

Patient	1 Before galactose and glutamic acid AAN_2	Gal	Gl	2 (15 min) AAN_2	Gal	Gl	3 (30 min) AAN_2	Gal	Gl	4 (45 min) AAN_2	Gal	Gl	5 (60 min) AAN_2	Gal	Gl	6 (90 min) AAN_2	Gal	Gl	7 Galactose Index
Bm	7 0	—	21	16 0	78	67	14 1	66	49	11 5	44	44	9 6	24	35	7 3	6	25	196
Cz	4 9	—	14	11 7	30	30	10 1	12	25	10 1	0	23	7 9	0	16	4 8	0	12	13
Wh	5 7	—	14	12 1	37	19	9 9	30	14	9 7	26	12	8 9	26	7	6 6	0	12	71
Og	6 0	—	35	11 0	26	51	9 7	22	25	8 4	7	32	7 4	4	23	5 6	0	25	35
Be	6 3	—	19	15 2	67	32	14 5	30	30	10 9	19	25	8 3	19	19	6 0	7	23	81
Co	4 5	—	16	15 0	74	14	11 0	41	21	9 4	19	23	6 6	19	19	5 7	7	7	144
Mn	4 5	—	21	11 3	74	21	10 1	52	25	8 5	37	12	6 6	33	9	5 3	7	21	183
Cl	3 9	—	12	12 0	115	19	11 1	74	16	10 2	52	19	8 7	37	19	7 6	30	12	277
Hu	5 5	—	12	14 1	74	28	13 1	52	23	11 9	33	21	9 3	26	16	7 7	19	16	248

Controls

Patient	8 Before galactose Gal	Gl	9 (15 min) Gal	Gl	10 (30 min) Gal	Gl	11 (45 min) Gal	Gl	12 (60 min) Gal	Gl	13 (90 min) Gal	Gl	14 Galactose Index
Bm	—	9	32	12	7	12	0	9	0	6	0	7	12
Cz	—	9	19	19	5	17	0	12	0	9	0	7	7
Wh	—	16	11	25	0	16	0	9	0	9	0	12	2
Og	—	30	19	35	7	33	7	31	0	33	0	21	8
Bo	—	19	33	12	15	9		9	0	9	0	12	25
Co	—	9	20	7	11	7	7	12	0	9	0	9	11
Mn	—	12	60	12	24	9	0	12	0	9	0	9	25
Cl	—	9	52	25	41	9	19	7	0	9	0	9	50
Hu	—	14	52	21	37	9	19	7	0	9	0	9	49

between insulin and circulating amino-acids mentioned in our introductory remarks   Of these adrenalaemia can, from our observations, be dismissed as an explanation   The second series of our experiments undertaken to test the effect of adrenalin fluctuations gave no confirmation of an interdependence of adrenalinaemia, as observed clinically, and amino-acid level in the blood   Moreover, the minor blood glucose variations well known as indicators of adrenalinaemia are not mirrored in the amino-acid values—an observation adding further weight to the clinical evidence

The inhibitory effect of insulin on proteolysis in the peripheral tissues may contribute to the lowering of the circulating amino-acids , but it is difficult to see how this should be linked up with the critical blood glucose level as closely as we found it   If an excess of insulin is responsible for the inhibitory effect such an excess is still present in our patients for some time after they have recovered from hypoglycaemia, as shown by their tendency to relapse into mild hypoglycaemia during the afternoon of the same day   Nevertheless, hypoamino-acidaemia does not persist after the blood glucose is above the critical level

The most plausible explanation is, therefore, that hepatic deamination for the purpose of glyconeogenesis is the main cause of the observed decrease of amino-acids—a process which one would expect to be controlled by the blood glucose level   The experiments of Laquet et al (1934), who found that in animals receiving small amounts of glycine insulin raised the blood amino-acid level if the blood glucose level was maintained by infusion of glucose, are strongly in support of such a control   So is the work of Ashby (1949) who has shown that the intraperitoneal administration of amino-acids to the adrenalectomised rat diminishes the rate of conversion of glucose to liver glycogen

To gain further insight into the interrelation between amino-acid circulating in the blood and the formation of glycogen was the object of our third experimental series   The great difference between the values for the   galactose index under the two sets of conditions shows that the rate of glycogen formation must be markedly affected by the presence of the excess amino-acid   The fact that the galactose persists in the circulation while the amino-acid concentration remains above normal is confirmatory evidence

The result of this as well as that of the earlier experiments can also throw some light on our primary problem, viz the waking effect of excessive amino-acids in hypoglycaemic coma   It has become clear why no direct relationship exists between the spontaneous hypoaminoacidaemia and the state of consciousness in hypoglycaemia , for at the blood glucose level of 80 mg /100 ml , at which hypoaminoacidaemia terminates, consciousness has been restored for a long time The brain, as explained before, returns to normal function at a much lower blood glucose level   Hence also the inefficacy of fluctuations of consciousness on the circulating amino-acids in a condition below the critical level (Table III)

The galactose experiments, on the other hand, suggest that the acute raising of the amino-acid level in the blood not only stimulates hepatic deamination of amino-acids, but at the same time slows down glycogenesis from circulating carbohydrates   Some of the circulating glucose which otherwise would be the main source of glycogenesis is thus saved   It may be that this glucose, although not raised in its actual level, persists in the circulation for a longer time and becomes more readily available to the brain cell—a hypothesis which will be tested in further experiments

SUMMARY

When plasma amino-acid nitrogen was observed before and after the injection of insulin in subjects undergoing Sakel's hypoglycaemia treatment, it was found to decrease as long as the blood glucose content was below 80 mg /100 ml If glucose was administered, this decrease was reversed only if the blood glucose rose above this critical level

The theory that adrenalin is responsible for the fluctuations in circulating amino-acids was not confirmed by clinical observations Serial experiments failed to show any relationship between behaviour of the subjects in hypoglycaemia and amino-acid level in the plasma

Hepatic deamination being one of the possible causes of hypoaminoacidaemia, galactose was used as an indicator of the interdependence of the level of circulating amino-acids and of glycogenesis from circulating carbohydrate Observations of its concentration and persistence in the blood of hypoglycaemic subjects was compared in the presence and absence of an excess of L-glutamic acid injected at the same time The results strongly suggested the existence of such an interdependence

The experiments are discussed considering previous findings of other critical blood glucose levels in hypoglycaemia, and in view of the explanation they may provide for the waking effect of excessive amino-acids in hypoglycaemic coma

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THE FAILURE OF ANTI-HISTAMINE DRUGS TO INFLUENCE THE LOCAL VASCULAR CHANGES IN EXPERIMENTAL BURNS.

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WHILST there is general agreement that the erythema of burns is due to the dilatation of the skin capillaries and venules, and that the oedema and blister fluids of more severe burns arise from the blood plasma via the walls of these tiny vessels, the mechanism of these phenomena is still in doubt   Ebbecke (1923) postulated that a chemical substance was liberated from damaged tissues and acted on the local capillaries   Lewis (1927) developed this theory and asserted that this compound was histamine or a histamine-like substance, the H-substance In more severe burns the oedema, blistering and peripheral extension of the ery-thema beyond the burn site, the " flare,' were regarded by Lewis as being due to the release of larger amounts of H-substance which acted both directly on the vessels and by stimulation of local axon reflexes   This theory could not be critically tested until drugs with a powerful anti-histamine action were discovered and experiments designed to test the truth of this hypothesis by the use of such drugs are recorded in this paper

PERMEABILITY AND BLOOD FLOW CHANGES IN GUINEA-PIG BURNS

The methods used in detecting these changes have been described elsewhere (Sevitt, 1949) and are summarized below

If the circulating plasma of an animal is dyed by the injection of Evans Blue (T1824) or Brilliant Vital Red (Colour Index 456) and a burn is inflicted, the development of a dyed patch of skin indicates the flow of blood through skin vessels of increased permeability   This has been called the *patch* reaction (Fig 1) and occurs in the less severe burns whether the dye is introduced before burning or some hours later   In more severe burns *stasis* of the dermal blood flow occurs and can be demonstrated by introducing the dye some time after burning   A narrow blue ring appears at the periphery of the burn, but the burn area itself remains erythematous   In this *ring-with-erythema* reaction, the persistant ery-thema which fails to blanche on pressure, indicates the presence of stasis, and the peripheral coloured ring indicates graded vascular damage at the edge of the burn

In some ring-with-erythema reactions, dye slowly moves inwards from the coloured peripheral ring, encroaching upon and obscuring the central erythema This is called the *temporary ring-with-erythema* reaction and indicates extreme retardation or *stagnation* of the blood flow

Double dye technique

Continuation of the blood flow over a period, or the development of stasis within a period, may be demonstrated by the introduction of the red and blue dyes at different times For examples two 20-seconds burns, at 60° and 65° C respectively, were inflicted on a guinea-pig An hour later Brilliant Vital Red was injected intracardially and red patch reactions developed in both burns (Upper and lower burns in Fig 1) Three hours later, Evans Blue was introduced In the 60° C burn, the red patch was replaced by a blue one and this indicated the continuation of the dermal blood flow and the continued permeability change over the four-hour period (Upper burns in Fig 3) in the 65° C burn, however, a narrow ring appeared at the periphery of the red circular patch which otherwise remained unchanged This indicated that stasis had occurred later than 1 hour after burning and within the following 5 hours (Lower right burn in Fig 3)

PRESENT INVESTIGATION

To determine whether anti-histamine drugs could modify their course, several burns varying in severity but of known temperature and duration were inflicted on each of a group of guinea-pigs Half of these were previously injected with one or other of the drugs β-dimethylaminoethyl benzhydryl ether hydrochloride (Parke Davis & Co), 2-phenylbenzyl aminomethyl-imidazoline (Ciba Ltd) or N-dimethylaminoethyl-N-p-methoxybenzyl-a-aminopyridine acid maleate (neo-antergan) (May & Baker, Ltd) These will be referred to as benadryl, antistin and anthisan respectively The permeability and blood flow changes of each burn were determined by the dye technique and the results were compared, burn for burn, in the two sets of animals Other comparisons made were the degree of oedema, the presence of erythema under the site of application of the burning iron and the development of cyanosis therein, the retention or loss of sensation in the burned skin and the subsequent depth of skin loss Four sets of experiments were performed and in none of them were important differences noted between the burns on the control and the anti-histaminized animals

EXPERIMENTAL

Albino guinea-pigs (560 to 670 g) were burned under open-ether anaesthesia by firmly pressing the burning surface of the iron on to the depilated back or abdomen The iron (Sevitt, 1949) was essentially a hollow brass cylinder through which circulated a current of hot water, the temperature of which could be varied and recorded The cylinder was closed at one end, and this, the burning surface, was circular and measured 1 in in diameter

Evans Blue and Brilliant Vital Red, both 1 per cent solutions, were injected intracardially in a dosage of about 1 c c

Sensibility was determined at intervals by pinching the burned skin with a forceps and noting the reaction of the animal

By examining the burns every 3 to 5 days, each was graded into one of the categories of no skin loss, superficial skin loss, partial skin loss or whole skin loss

CONTROL OF THE EXPERIMENTS

Preliminary experiments with, and dosage of, anti-histamine drugs.

When 0 05 c c of 0 05 per cent histamine acid phosphate was injected into the depilated skin of a normal guinea-pig which had previously been injected intracardially with Evans Blue, a well marked blue wheal 9 to 12 mm in diameter occurred   This procedure was adopted as the routine skin histamine test

Half to one hour after the intramuscular injection of 50 mg of antistin (70 to 90 mg per kg) into a guinea-pig the skin histamine test failed to produce a wheal and the inhibitory effect generally lasted 3 to 4 hours

Benadryl in a 30 mg dose produced complete protection, but made the animals agitated, restless and unsteady   In a dose of 10 mg (15 to 17 mg per kg) toxic signs were absent, but the protection was not complete, the blue wheal being reduced to a minimum of 3 to 4 mm in about half an hour

The maximum non-toxic dose of anthisan which produced complete skin protection against histamine for 3 to 4 hours was between 12 5 and 25 mg , the latter dose producing slight restlessness and agitation   A dose of 17 5 mg (25 to 30 mg per kg) was non-toxic and was equally effective over the same period

During these tests, a blue staining 1 to 2 mm round the puncture site occurred after some histamine tests, even during a period of apparently complete anti-histamine protection   As similar " needle-track reactions " often occurred in both anti-histaminized and control animals after any needle puncture, the phenomenon was regarded as unrelated to the histamine injection and probably of mechanical origin

On the basis of these results antistin in a dosage of 50 mg per guinea-pig was used in Experiments 1 and 2, 10 mg of benadryl in Experiments 3, and 17 5 mg of anthisan in Experiment 4

Control of anti-histamine action in main experiments

In Experiments 1, 3 and 4, a guinea-pig which was not burned was introduced into each experiment in order to determine the efficiency of the anti-histamine drug given to the other animals   Evans Blue was injected intracardially, then a histamine skin test was performed and 10 to 20 minutes later the appropriate anti-histamine drug was injected intramuscularly in the same dosage as used in the burned animals   Histamine skin tests were then performed at 1/2 to 1 hourly intervals

In Experiments 1 and 4 these were completely or almost completely negative for 4 to 5 hours , in Experiment 3, the blue wheal was reduced from 11 mm in

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EXPLANATION OF PLATES

Fig 1 — Control animal (C₁)   Brilliant Vital Red injected before burning
Fig 2 —Anthisanised animal (A₁)   Brilliant Vital Red injected before burning
Fig 3 —Control animal (C₁)   Evans Blue injected 3 hours later
Fig 4 —Anthisanised animal (A₁)   Evans Blue injected 3 hours later

Figs 1–4 —(See text, Experiment 4) show the similar capillary permeability and blood flow changes occurring in four similar burns inflicted on the abdomens of two guinea pigs, one anthisanised, A₁, the other a control animal, C₁

Note   The contrast between the red and blue colours on the skin is not so marked in the black and white reproduction as it was *in vivo*

1

3

2

4
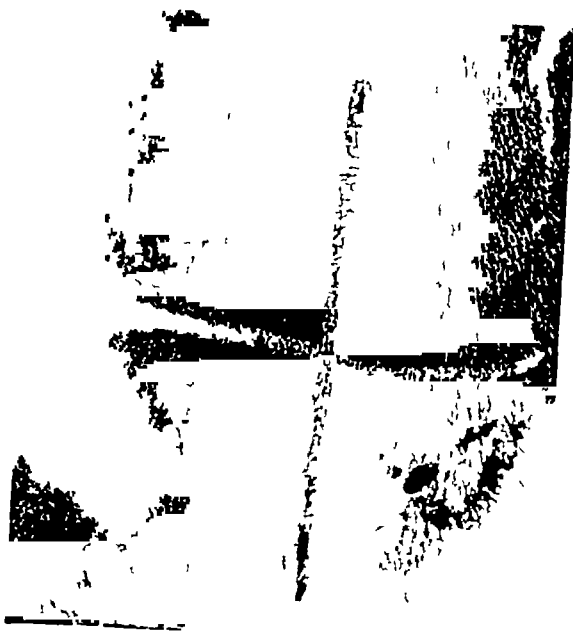
diameter before injection of benadryl to a minimum of 5 to 4 mm, the partial protection lasting for longer than 3 hours. In Experiment 2, the "anti-histamine control" animal was not used, but the initial 50 mg of antistin was supplemented by an additional injection of 12 5 mg half an hour after burning

In Experiments 1, 2 and 3 skin histamine tests on all the burned animals were carried out at various times after burning with the intention of directly determining the efficiency of the anti-histamine action in the burned, anti-histaminized animals. Unexpected difficulties in interpretation arose. All the anti-histaminized animals gave the expected weak or negative reactions, and most of the control guinea-pigs showed repeated positive skin reactions, but unexpected weak or negative results were obtained in three of these animals. For example, in one *unprotected* animal a skin histamine test produced a well marked wheal soon after burning, 1 and 2 hours later similar tests were negative, but after another 2 hours were again positive. In other words, this burned animal became temporarily insensitive to histamine. This result is difficult to explain. However, whatever the explanation of this phenomenon is, it was concluded that when negative skin histamine tests occurred after burning an anti-histaminized animal, the failure of the skin to respond to injected histamine was not necessarily due to the action of the injected anti-histamine drug

Nevertheless, as a result of the histamine tests on the *unburned* anti-histaminized animals, we may conclude that in Experiment 1, 2 and 4, the burned anti-histaminized animals were completely protected against the effects of a relatively large dose of histamine and that in Experiment 3 the protection was partial

MAIN EXPERIMENTS

Experiments 1, 2 and 3

The general plan of Experiments 1, 2 and 3 is shown in the Table I. At least four animals, A, B, C and D, were used in addition to the unburned "anti-histamine

TABLE I —*Showing the General Plan of Experiments* 1, 2 *and* 3

Order of technique used	Guinea pig				
	A *	B *	C	D	Un burned control
Evans Blue injected intracardially	+	+	−	−	+
Anti-histamine drug injected intramuscularly	+	−	+	−	+
Repeated histamine skin tests					+
Animals burned	+	+	+ interval	+ interval	−
Evans Blue injected intracardially	−	−	+	+	−

* In Experiment 2 duplicate animals used

control" previously mentioned. In Experiment 2, duplicate animals A and B were used. A and B were injected with Evans Blue, and a few minutes later A and C received the appropriate anti-histamine drug intramuscularly. In Experiments 1 and 2, histamine skin tests were performed on animal A 1/2 to 1 hour later and produced no reactions

Similar burns were then performed on corresponding parts of the backs or abdomens of all the animals   At repeated intervals a clinical estimate of the burn oedema was made (0, +, + + or + + +) and skin sensation tests were performed In A and B the changes in the burns to circulating dyed plasma was noted, and in C and D the presence or absence of cyanosis in the burn erythema was looked for Such peripheral cyanosis indicates local slowing of the blood stream (Lundsgaard and Van Slyke, 1923)   At definite times after burning Evans Blue was injected into animals C and D, these intervals being 3, 3/4 and 1 hours in Experiments 1 2 and 3 respectively   The reactions of the burns to plasma dyed at this stage were noted   Most of the animals survived and the depth of skin loss in each burn was clinically determined

Choice of burns —On the bases of previous experience (Sevitt, 1949) the temperature and duration of the burns inflicted were so selected as to give a complete range of severity of burning up to, but not including burns which produced immediate heat necrosis of the whole skin   The burns ranged in severity from those in which the capillary permeability changes and subsequent skin loss were absent or slight and in which sensation was unimpaired, to those in which rapid stasis of the local blood flow and analgesia occurred and which were followed by whole thickness sloughing of the skin

In Experiment 1, four burns at 56° C and four at 62° C for 10, 20, 30 and 60 seconds respectively were performed on each animal   In Experiment 2, only two burns at 55° C for 20 and 30 seconds were inflicted, and in Experiment 3 the burns in increasing order of severity were 54° C for 20 seconds, 58° C for 10 seconds and 60° C for 20, 30 and 60 seconds

Experiment 4

In this experiment anthisan (17 5 mg per animal) was the anti-histamine drug used , the permeability and blood flow changes in the burns were determined by the double-dye technique

Five albino guinea-pigs were used, one of which was not burned and served as the " anti-histamine control "   The other four (A$_1$, A$_2$, C$_1$ and C$_2$) were paired off, each pair receiving identical treatment, that is to say, each set of observations was duplicated   All were injected intracardially with Brilliant Vital Red   A$_1$ and A$_2$ then received intramuscular injections of anthisan, C$_1$ and C$_2$ serving as the controls   Half an hour later a series of six burns, all at 61° C , were inflicted on each animal   The severity was varied by using different burning times , burns of 5, 10, 20 and 30 seconds' duration were made on the abdomens, and burns of 45 and 60 seconds were made on the backs   Three hours after burning Evans Blue was injected into all four animals

RESULTS

In none of the experiments was any significant difference noted between comparable burns on the control and anti-histaminized animals   In particular no significant differences were observed either in the erythema, capillary permeability or blood flow changes in the burns, in the degree of oedema occurring, in the sensory changes or in the subsequent depths of skin loss   For this reason only the results of Experiment 4 are recorded in detail

Results of Experiment 4

The reactions of the abdominal burns in one of the control and one of the anthisanized animals (C₁ and A₁ respectively) are shown photographically in Fig 1 to 4, where the upper left, upper right, lower left and lower right burns were of 5, 10, 20 and 30 seconds' duration respectively

In Fig 1 and 2, which were taken 1 hour after burning, the reactions of the burns to the presence of circulating red-dyed plasma are shown   All the burns in all the animals developed patch reactions, which indicated that the local blood flow was continuing in each burn and was passing through minute dermal vessels of increased permeability   In the 5 seconds' burns, however, the patch reactions (and hence the permeability change) was delayed and less marked than in the remainder   Oedema was not detected in any of the 5 seconds' burns, was very slight or absent in the 10 seconds' burns, but was well marked in all the burns of longer duration   No significant differences were noted, burn for burn, between the control and anthisanized animals during this period

The reactions of the abdominal burns in Animals A₁ and C₁ to the Evans Blue injected 3 hours later are shown photographically in Fig 3 and 4   In A₁ a blue patch failed to appear in the 5 seconds' burns, the slight red patch remaining unchanged   This indicated that the slightly increased permeability change of the dermal vessels detected 3 hours previously had now disappeared   In animals A₂, C₁ and C₂, however, blue patches appeared, but were not prominent (Top left burn in Fig 3)   On the other hand, definite and well marked blue patch reactions occurred in all the 10 seconds' burns (Top right burns in Fig 3 and 4), indicating the continued circulation of the local blood flow and increased capillary permeability change in each of these burns for a period of three hours after burning   In all the 20 seconds' burns, blue rings appeared at the periphery of each red patch, but within half an hour Evans Blue was entering and obscuring each of these red-dyed areas (Lower left burn in Fig 3)   These reactions are of the same nature as temporary ring-with-erythema reactions and indicate extreme retardation or stagnation of the local blood flow   In all the 30, 45 and 60 seconds' burns blue peripheral rings also appeared, but these remained unchanged   Stasis of the dermal blood flow in each burn had occurred

No real differences were noted, therefore, in the permeability and blood flow changes occurring, burn for burn, between the control and anthisanized animals Skin sensation was normal in the 5 and 10 seconds' burns   In the 20 seconds' burns sensibility varied with time, that is to say, normal or partial sensibility was present soon after burning, but within a few hours partial or complete analgesia developed   In the 30, 45 and 60 seconds' burns, analgesia was complete within an hour or two of burning   The sensory differences were no more marked burn for burn, between the control and anthisanized animals, than between the two controls or between the anthisanized animals themselves

Three of the animals (A₂, C₁ and C₂) survived   The degree of skin loss in the 5 seconds' burns was very slight in each burn , the skin loss was superficial in all the 10 seconds' burns , and in the 20, 30, 45 and 60 seconds' burns whole skin loss developed in all three animals

CONCLUSION

Within the limitations of the experimental methods used, the protection of guinea-pigs against histamine by the drugs benadryl, antistin or anthisan has no

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detectable effect on the changes produced by burns of different degrees of severity
These changes include erythema of the skin, the presence or absence of per-
meability change developing soon after burning or present some time later, the
development of stagnation or stasis in the dermal blood flow, and the degree of
oedema formation In addition, retention or loss of sensibility to a painful
stimulus was not affected nor was the subsequent clinical course of the burn

DISCUSSION

The symptoms of anaphylaxis and related disorders and the local reactions
of the body to trauma were explained by Dale (1929), and Lewis (1927) as being
due to the liberation of intracellular histamine In particular, Lewis postulated
that when the skin was injured in various ways H-substance was released from
the epithelium and acted upon the underlying minute vessels in the dermis In
this way dilatation of the capillaries and venules was produced and this was
followed by the exudation of fluid when sufficient H-substance was liberated

These theories were simple in concept and had the advantage of explaining a
multitude of biological phenomena on the basis of the known pharmacological
action of a single chemical compound Moreover, the histamine concept led to
a successful search for drugs capable of opposing the action of histamine, and the
discovery of these drugs led to their use in experiments and clinical trials, one
of the purposes of which was to test the truth of the histamine hypotheses of
Dale and Lewis The results of these have varied on the one hand anti-hista-
mine drugs protect a sensitized animal from many of the symptoms of anaphy-
laxis (Yonkman, Oppenheimer, Rennick and Pellet, 1947) and there is general
agreement that they are effective against urticaria (Schindler, 1946, Hunter,
1947, Overton, 1948), and against hay fever and other forms of spasmodic rhinitis
(Southwell, 1948) It would appear, therefore, that the pharmacological action
of liberated histamine plays a dominating part in the production of these conditions

On the other hand, spasmodic asthma rarely responds to anti-histamine drugs
(Southwell, 1948) Moreover, the prophylactic use of anti-histamine drugs is
unable to suppress or modify the Arthus phenomenon in horse-serum sensitized
rabbits (Last and Loew, 1947, Dammin and Bukantz, 1949) Furthermore, the
reaction of the rabbit's skin vessels to the intradermal injection of horse-serum,
trypsin, snake venom, staphylococcus toxin, tetracaine, iodine and heparin are
not influenced by the prophylactic injection of benadryl or neoantergan (Last and
Loew, 1947) The experiments now reported have shown that neither benadryl,
antistin nor anthisan can inhibit or modify the permeability, vascular flow and
other changes in the skin of guinea-pigs subjected to burns of different degrees of
severity

There is evidence, however, that not only is histamine liberated from burned
tissues, but new formation of histamine may occur (Dekanski, 1945) It follows,
therefore, that the ineffectiveness of anti-histamine drugs in modifying the
reactions produced by burning does not mean that histamine is not liberated from
or not formed in burned tissues, but means that the problem is a much wider
one than discovering in which biological reactions histamine is liberated or
excreted In other words, the finding that histamine is released does not neces-
sarily mean that it is responsible for the reaction present, even when this resembles
one of the pharmacological actions of histamine In anaphylaxis, urticaria and

hay fever it would appear that the internal release of histamine plays a major part, but histamine release cannot be very important in the response of the skin vessels to heat

Another explanation for vascular changes occurring in burns has been put forward (Cullumbine and Rydon, 1946) This is based on the discovery of leucotaxine in inflammatory exudates by Menkin (1940) Intradermal injection of this substance in high dilution causes an increase of capillary permeability and attracts leucocytes to the affected area Cullumbine and Rydon have found leucotaxine in the vesicle fluid of heat and mustard burns The leucotaxine theory fundamentally resembles that of Lewis and is subject to similar criticisms In the absence of a substance antagonistic to leucotaxine this theory cannot be subjected to a critical trial

The cause of the vascular changes in burns remains unknown, though it is possible that heat itself is the direct noxious agent

SUMMARY

The protection of guinea-pigs against the action of histamine by the drugs benadryl, antistin or anthisan (neoantergan) had no detectable effect on experimental burns of various degrees of severity The changes investigated included the erythema of burns, the capillary permeability and qualitative blood flow changes in the skin, the clinical degree of oedema formation, the skin sensibility and the subsequent clinical course of the burns

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THE INTRACELLULAR LOCALIZATION OF β-GLUCURONIDASE

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Received for publication September 25, 1949

It has been suggested that the activity of the enzyme β-glucuronidase reflects the degree of cellular proliferation taking place in a tissue or organ (Levvy, Kerr and Campbell, 1948 ; Keir, Campbell and Levvy, 1949) Evidence has been presented showing that the enzyme activity of tissue extracts is increased in damaged organs undergoing repair, during liver regeneration subsequent to partial hepatectomy, and in the organs of young mice as compared with adults

Biochemical assay was necessary in order to obtain a quantitative expression of β-glucuronidase activity, but this gives no indication of the actual site of the enzyme in the cell The recent publication of two histochemical methods (Friedenwald and Becker 1948), now enables the location of the enzyme to be studied

EXPERIMENTAL

The methods used for the histochemical detection and localization of β-glucuronidase were essentially those devised by Friedenwald and Becker (1948) Their technique depends upon the hydrolysis at 37° C of a conjugated glucuronide by the enzyme in fresh frozen sections of tissues, with either the direct liberation of an insoluble azo dye, or the formation of a precipitate of ferric hydroxyquinoline which is converted into Prussian blue In both methods microscopically visible substances are deposited at the presumptive site of enzyme activity in the tissue

(1) " 1-*ortho-hydroxyphenylazo-2-naphthol glucuronide* ' (Friedenwald and Becker, 1948) The substrate solution was made up by dissolving 50 mg of the glucuronide in 1 litre 0 1 N acetate buffer at pH 5 with warming When cool, the solution was filtered Unfixed frozen sections, between 20–40μ in thickness, were incubated in a water bath at 37° C in 100 ml substrate solution in stoppered vessels of such capacity that the air space above the solution was small

After incubation, the sections were washed in distilled water, fixed in 10 per cent formalin and 1 per cent acetic acid, and either mounted directly in glycerine jelly, or counterstained with 0 5 per cent methyl green in 0 5 per cent acetic acid and then mounted

Controls for the specificity of the method consisted of incubating the substrate alone, incubating with inert foreign matter, e g paper pulp, and incubating with frozen sections of boiled mouse kidney In no case did hydrolysis occur, even after 18 hours

(2) 8-*hydroxyquinoline glucuronide* (Brahm, 1899) The substrate solution was prepared as described by Friedenwald and Becker (1948) After the addition

of 0 03 N ferric chloride, followed by incubation at 37° C for 2 hours, it was found necessary to carry out a double filtration through Whatman No 40 paper to remove the very fine suspension of ferric hydroxyquinoline which formed Filtration was carried out in an incubator saturated with water vapour

It was found advantageous to place the frozen sections in chilled 0 1 N acetate buffer pH 5, as soon as they were cut and to remove as much buffer as possible by touching the sections against a Whatman No 3 paper before transferring them to the substrate Small weighing bottles capable of holding about 6 ml were used as incubation vessels These had the advantage that there was only a small air space between the liquid surface and the stopper

The substrate was used on the day it was prepared, and sections were placed in it within 2 to 3 minutes of cutting One of the main difficulties associated with this method was to obtain a complete conversion of the enzymatically liberated ferric hydroxyquinoline to Prussian blue This was largely overcome by increasing the concentration of potassium ferrocyanide from 1 per cent to 2 per cent in the ferrocyanide-hydrochloric acid mixture Sections were fixed as in method (1) and counter-stained with basic fuchsin 1   5000 in 1 per cent acetic acid

RESULTS

Location of the enzyme

Fig 1 shows that the enzyme is localized to the cortical region of the male mouse kidney and Fig 2 is an adjacent section incubated in a control solution in which 8 hydroxyquinoline glucuronide was replaced by an equal volume of buffer A higher magnification (Fig 3) shows the enzyme to be mainly localized to the proximal convoluted tubules In suitably thin sections at a still higher power the enzyme appears to be localized in the basal parts of the cells constituting the proximal convoluted tubules, coincident with the parallel rods frequently to be seen in ordinary histological preparations These rods are thought to be mitochondria (Maximow and Bloom 1948) The distal convoluted tubules do not show such an intense reaction and there is only a very slight staining of Bowman's capsule and the glomerulus of the male kidney (Fig 5 and 6) Similar localization to the mitochondria was seen in frozen sections of liver (Fig 7)

Paraffin sections of tissue fixed according to the Gomori technique for phosphatase, showed only a very faint staining with 1-ortho-hydroxyphenylazo-2-naphthol glucuronide ' and none at all with 8-hydroxyquinoline glucuronide

Influence of pH

In a series of trials with both glucuronides in which the pH of the substrate was respectively 4 5, 5, 5 2 and 5 5, the best results were obtained within the range 5–5 2 using the normal male mouse kidney

Inhibition by saccharic acid

Saccharic acid is known to be an effective inhibitor for β-glucuronidase *in vitro* (Karunairatnam and Levvy, 1949) Its action was utilized to test the specificity of the histochemical methods Potassium hydrogen saccharate was dissolved in water to give a concentration of 0 1 M Before final adjustment

of the volume the pH was adjusted to 5 (glass electrode) This solution was added during preparation of either substrate solution in place of part of the acetate buffer

Using 8-hydroxyquinoline glucuronide as substrate, 0·001 M saccharate caused almost complete inhibition of hydrolysis by a section of male mouse kidney (Fig 4) Fig 4 should be compared with Fig 3 which shows another section from the same kidney incubated with substrate in absence of saccharate

To see whether saccharic acid penetrates intact cells, a bisected mouse kidney was incubated overnight in 8-hydroxyquinoline glucuronide solution containing 0 001 M saccharate As control, the other kidney, also bisected, was incubated in substrate alone Serial frozen sections from the control kidney showed penetration of the glucuronide to a depth of 20 to 30 cells Inhibition by saccharate was almost complete at all depths

Using " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide " as substrate, inhibition by saccharate was not pronounced, although the inhibitor appeared to delay the reaction A more pronounced inhibition resulted when sections were incubated with 0 001 M saccharate before placing in the substrate and inhibitor With this glucuronide it appears that the insolubility of the reaction product is so great that the reaction is governed more by the rate of diffusion of the substrate than by the amount of enzyme available After 2 or 3 hours incubation the fullest possible intensity of staining is seen, independently of the activity of the enzyme present At shorter periods, some discrimination in intensity is possible between different preparations, but the reaction is not very suitable for comparative work

Influence of sex on results

It was incidentally observed that male mouse kidneys gave a more intense staining reaction to both methods than female organs A suitably short period

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EXPLANATION OF PLATES
(All photographs prepared from frozen sections)

PLATE I

Fig 1 —Longitudinal section of male mouse kidney showing localization of β glucuronidase to the cortex Substrate 8 hydroxyquinoline glucuronide Incubation time 12 hours × 12

Fig 2 —Control section incubated for the same time in the absence of the glucuronide × 12

Fig 3 —Localization of the enzyme mainly to the proximal convoluted tubules of the kidney Substrate 8 hydroxyquinoline glucuronide Incubation time 12 hours × 36

Fig 4 —Succeeding section to that shown in Fig 3 Hydrolysis has been inhibited by 0 001 M saccharate × 36

PLATE II

Fig 5 —Proximal convoluted tubules of the kidney, showing localization of staining to the mitochondria Substrate ' 1 ortho hydroxyphenylazo 2 naphthol glucuronide " Incubation time 80 minutes × 300

Fig 6 —As for Fig 5 The glomerulus is unstained Incubation time 100 minutes × 300

Fig 7 —Specific staining of the mitochondria of mouse liver cells Substrate " 1 ortho-hydroxyphenylazo 2 naphthol glucuronide " Incubation time 80 minutes × 300

Fig 8 —3 4 benzpyrene induced squamous cell carcinoma of skin of mouse, showing intense staining of the malignant cells growing down into the corium Substrate 8 hydroxyquinoline glucuronide Incubation time 15 hours × 36

Fig 9 —Granular and filamentous mitochondria staining specifically in the G R C H /15 chicken fibrosarcoma Substrate 8 hydroxyquinoline glucuronide Incubation time 10 hours × 300

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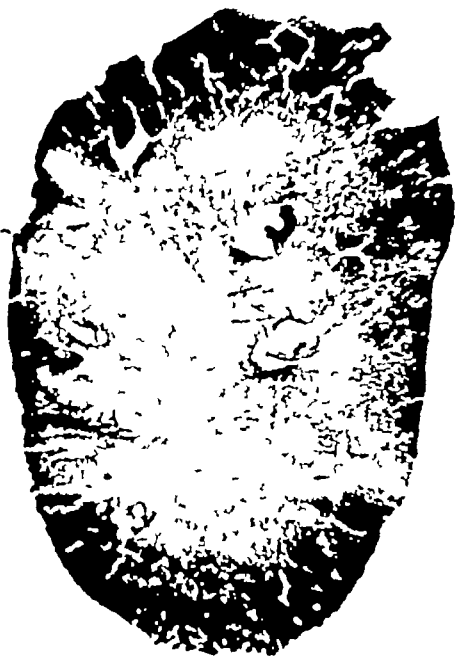
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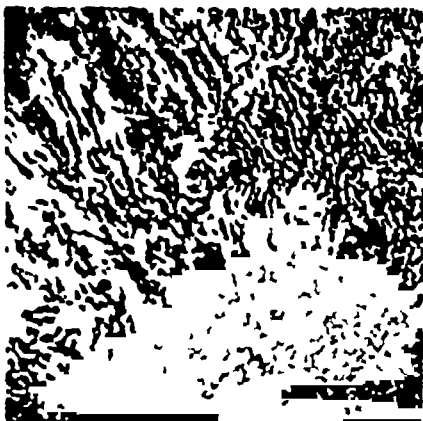
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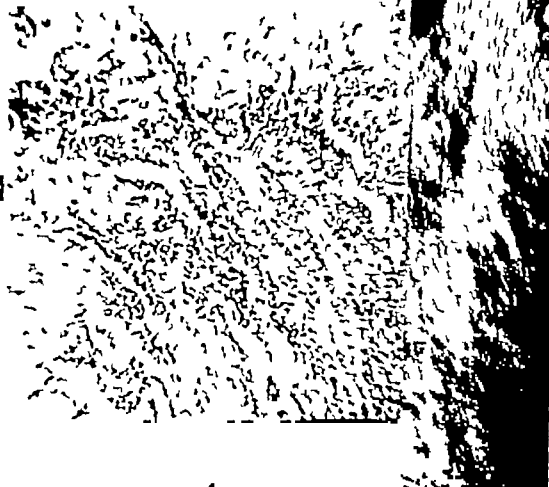
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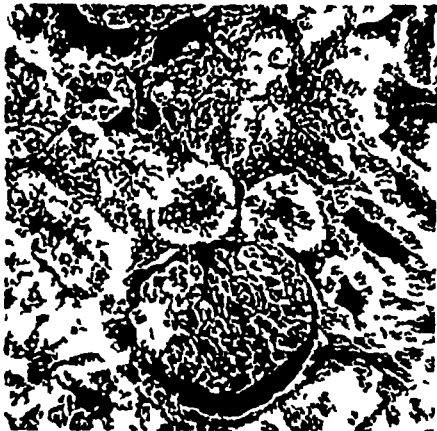
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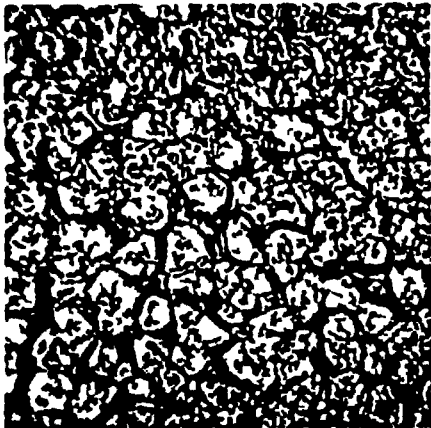
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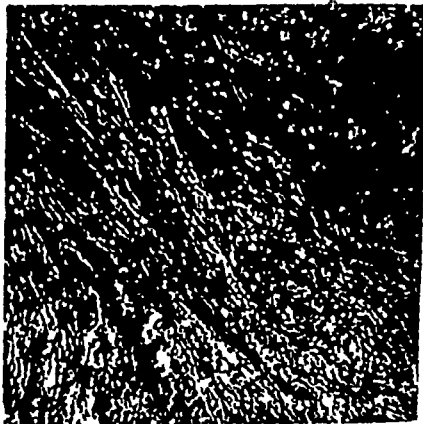
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of incubation was used in Method 1. The hydrolytic reaction was reduced in castrate male mouse kidneys, and increased in kidneys from ovariectomized mice, in both cases 8 weeks subsequent to operation   Localization of the enzyme was identical in male or female kidneys

Results with tumour tissues

Great difficulty was experienced in obtaining satisfactory histochemical pictures using rapidly-growing malignant tumours, because of frequent widespread necrotic changes, while in certain growths the presence of large amounts of mucoid material interfered with the reaction   However, although results were often unsatisfactory from the photographic aspect, a fair picture of the distribution of the enzyme was obtained in a preliminary study   As a whole, tumours seemed high in enzyme activity compared with normal liver and kidney   Table 1 summarizes the findings in those tumours examined

TABLE I

Tumour	Enzyme distribution
Rous sarcoma, fowl	Scattered discretely in cytoplasm
G R C H /15 fowl	,, ,,
Ovarian adenocarcinoma fowl	Traces of enzyme confined to glandular epithelium None in schirrious areas
Mouse mammary carcinoma	Rich in anaplastic areas, none in blood cysts, degenerated parts or connective tissue
Squamous cell carcinoma, skin of mouse	Rich in poorly differentiated downgrowing epithelium Traces in dermis
Papilloma, skin of mouse	None

Assessing these preliminary observations, it may be concluded that β-glucuronidase is most rich in those parts of tumours where cell division is a prominent feature   This is especially well shown in Fig 8 where a very pronounced staining is practically confined to the down-growing malignant epithelial cells of a mouse skin epithelioma induced by an injection of 3   4 benzpyrene As in normal tissues, suitable preparations show the enzyme to be confined to the cytoplasm of tumour cells (Fig 9)

DISCUSSION

Fishman (1940) showed that the prolonged administration of menthol to mice caused a rise in the activity of β-glucuronidase in the liver, kidney and spleen   He postulated that this enzyme acts synthetically, but this has not yet been established and other workers take the view that the function of the enzyme in the animal body is purely hydrolytic (Levvy, 1947 , Karunairatnam, Kerr and Levvy, 1949)

In a later report, Fishman and Anlyan (1947) demonstrated a high β-glucuronidase content in some human tumours   It is interesting to note that

in Table I of their communication, the activity of the enzyme was closely cor-
related with the clinical malignancy of the tumour   For example, benign tumours
showed a low activity, tumours such as schirrous carcinoma also low, whilst
adenocarcinoma and medullary carcinoma had high enzyme activities   It
is difficult in cancer studies to obtain the corresponding normal tissue unless
an organ like the liver is involved, and a serious criticism may be made of the
normal tissue controls used by Fishman and Anlyan (1947)   Whereas an organ
may be a complex of tissues, only one of these is usually involved in neoplasia
The fact remains, however, that striking elevations of the enzyme figures occurred
in malignant tumours compared with normal tissues or organs from which those
tumours arose   This work has been confirmed and extended by Odell and Burt
(1949)

Kerr and Levvy (1947) at the same time as Fishman's work on tumours,
showed that a rise in enzyme activity occurred in mouse liver or kidney following
administration of toxic agents not known to form glucuronides, dependent on
the site of damage   The action of menthol, which was found to be a liver and
kidney poison could be explained on the same terms   Kerr, Levvy and Campbell
(1947) found that the rise in the enzyme activity was not detectable in the early
stage of poisoning, as, for example, with mercuric nitrate or chloroform in the
kidneys of the male mouse, but occurred as soon as repair was histologically
demonstrable   Furthermore, sub-total hepatectomy of mice, either by ligation
or cautery, caused rapid hypertrophy of the remainder of the organ, a great deal
of mitotic activity and an elevation of β-glucuronidase activity   It was also
found that the liver, spleen and kidney of baby mice showed much higher enzyme
levels than in adults   This was also true of uterus (Kerr, Campbell and Levvy,
1949)

It has been shown by Crabtree (1941) that there is a morphological difference
in the cells of Bowman's capsule in male and female adult mice   Eschenbrenner
and Miller (1945) demonstrated the specific toxicity of chloroform for the con-
voluted tubules of the male mouse kidney   Furthermore they showed that the
epithelium of Bowman's capsule in castrated male mice tended to change to the
female type, and that the kidneys of such mice were no longer susceptible to
chloroform poisoning   These findings were confirmed (Kerr, Campbell and
Levvy, 1949) and it was also shown that a rise in β-glucuronidase occurred in
male kidneys subsequent to the administration of chloroform, but not in female
kidneys or kidneys of castrated male mice, and the rise, when demonstrable,
was coincident with repair processes in the damaged organ

Further evidence for a relationship between cell proliferation and β-glucuroni-
dase activity appears to be provided by Fishman and Fishman's (1944) obser-
vation that the administration of oestrogens to ovariectomized mice causes
a rise in uterine glucuronidase, their explanation being that glucuronidase is
primarily concerned with the conjugation of oestrogen   A more likely explana-
tion would be on the basis of proliferating endometrium   The effect can be
prevented by the concurrent administration of androgen (Kerr, Campbell and
Levvy, 1949)   Odell and Burt (1949) consider that the association between
enzyme activity and proliferative activity provides the best explanation of their
results for human cervical cancer, and those conditions characterized by an
increased rate of cell division such as pregnancy and the proliferating corpus
luteum

The first part of the present paper deals with preliminary findings using histochemical methods for the localization of β-glucuronidase The association with mitochondria is perhaps not unexpected, as it does not seem unreasonable to assume that many intracellular enzymes must be associated with these organoids which in the living cell are in constant movement, exhibit changes of form, and frequently show a well marked tendency to polarity, all indicative of intracellular activity

It is significant that the enzyme is confined to that part of the kidney parenchyma, the cortex, which is damaged in chloroform poisoning

The experiments with saccharate suggested that the test with 8-hydroxy-quinoline glucuronide at least is specific for glucuronidase It is interesting to note that the experiments give unequivocal evidence of penetration of saccharic acid into the intact cell The failure of saccharic acid to influence glucuronide synthesis by surviving liver slices (Karunaratnam and Levvy, 1949) thus provides excellent grounds for believing that β-glucuronidase is not involved in the synthesis of glucuronides *in vivo* In the few histochemical observations made to date and described above, β-glucuronidase appears to be particularly rich in actively growing tumours or parts of tumours

SUMMARY

Using techniques devised by Friedenwald and Becker (1948) the localization of β-glucuronidase has been studied in normal mouse kidney and liver, and in several chicken and mouse tumours In the kidney, enzyme activity is practically confined to the proximal convoluted tubules in the cortex

The intracellular site of the enzyme in both liver and kidney appears to be coincident with the mitochondria

The hydrolysis of 8-hydroxyquinoline glucuronide, by fresh frozen mouse kidney sections is inhibited by 0 001 M saccharic acid Serial frozen sections of a bisected kidney treated in the same manner, show penetration of the cells by saccharic acid, as indicated by inhibition of hydrolysis

In unit time, the male mouse kidney shows a stronger staining reaction in these histochemical methods, than the corresponding female organ Castration of males diminishes this effect, whereas ovariectomy augments it, when compared with the enzymatic activity of kidneys from intact animals of the like sex

In a preliminary study the enzyme activity is found to be high in malignant tumours, and is mainly localized in anaplastic areas where cell division is prominent

The results obtained by a number of workers can be interpreted in terms of a relationship between β-glucuronidase activity and proliferative activity

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THYROID-PARATHYROIDECTOMY IN PARABIOTIC RATS

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Tumour formation in the rat thyroid under the influence of goitrogens has been attributed to the continuous stimulation of the thyroid cells by excessive amounts of thyrotropic hormone (Griesbach, Kennedy and Purves, 1945; Money and Rawson 1947) It seemed desirable, however, to exclude the possibility that the goitrogenic agents apart from causing indirectly the release of large amounts of thyrotropic hormone, had a direct carcinogenic effect on the thyroid cells The induction of thyroid adenomata in the rat under conditions of extreme iodine deficiency, leading to thyrotropic stimulation and consequent hyperplasia of the thyroid, has been considered to have proved the fact that a physiological agent acting intensively for a long time, can produce neoplastic cell changes It occurred to us that the method of parabiosis might provide a means of stimulating the thyroid without introducing a foreign chemical agent or subjecting the animal to a state of thyroxine deficiency It is well known that after castration of one parabiotic partner large amounts of gonadotropic hormone released from the castration pituitary produce marked hyperplasia and hypertrophy of the other partner's gonads The sex hormones produced by the intact partner are so rapidly destroyed that the amounts transferred to the castrated partner are insufficient to inhibit this animal's production of gonadotropic hormones (Zeckwer 1946) If a similar situation occurred after thyroidectomy the thyroid of the intact animal would be subjected to a continuous stimulation by the thyrotropic hormone from the thyroidectomized partner Necessary conditions would be (a) rapid destruction or excretion of thyroxine by the intact partner, and (b) the passage of sufficient amounts of thyrotropic hormone across the junction from the thyroidectomized animal Naiko and Ikonen (1934), in rats, did not find any changes in the thyroid when parabiosis between a normal and a thyroidectomized animal was performed This result has been confirmed and, in addition, changes in the pituitary and parathyroid are described

METHODS

The animals used were rats of a Wistar strain, 6 to 10 weeks old Litter mates of the same sex were joined in parabiosis, with open abdominal junction, and three weeks later the right partner of pairs which had shown satisfactory growth was thyroidectomized

The diet fed was free of meat protein, and has been described in a previous publication (Diet S3, Griesbach, Kennedy and Purves, 1945) The iodine content of this diet was low and a number of pairs was supplied with additional iodine in the drinking water (1 μg I_2 as KI per ml) One pair (No 1) received no additional iodine; pairs 6 to 11 were provided with approximately 10 μg daily

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throughout the experiment, and pairs 2 to 5 received this supplement during the first weeks, but it was withdrawn for the last 104 days

The animals were killed 15 to 233 days after thyroidectomy   The pituitaries were fixed in sublimate formalin, and stained according to Martin s modification of Mallory's method   The thyroids were fixed in formol saline and the cell heights measured as described by Griesbach and Purves (1943)

<div align="center">RESULTS</div>

Growth

In ten of the eleven pairs the growth of the thyroidectomized animal was comparable with that of the intact partner   The maximum weight reached by a pair was 444 g

Thyroids

The weights and mean acinar cell heights of thyroids of pairs 6 to 11 which received iodide throughout the experiment were normal (10 mg per 100 g and 7 1μ)   In the group which received additional iodide during the early part of the experiment only, the weights and mean cell heights were increased (average 13 4 mg per 100 g and 10 1μ)   The thyroid of pair No 1 was enlarged (28 4 mg per 100 g)

Pituitaries

(1) Thyroidectomized partners (R)   Acidophil cells were present in all pituitaries, but in slightly lower numbers than in our normal animals, which have an average value of 48 per cent   In ten animals the number of basophil cells was high and typical thyroidectomy changes were present   One rat, No 5, did not have an increased number of basophils although no thyroid could be found at autopsy

(2) Intact partners (L)   The pituitaries of these partners showed well-granulated acidophil cells, the percentage of which was higher in the group receiving a continuous iodide supplement   Rat 1, which received no additional iodide had a definite basophilia, Rat 2 a slight basophilia while the others showed normal figures   These results are summarized in Table I

Gonads

The two pairs of female rats had normal oestrous cycles   Both partners gave birth to normal litters

Parathyroids

The parathyroids of all the intact partners were enlarged   Microscopically, they showed a high activity   The cells were enlarged, the nuclei were vesicular and poor in chromatine   there was an abundance of clear cells with eccentric nuclei   The dark cells were scarce and were found only at the periphery (Fig 1 and 2)

The blood calcium content examined in five pairs was not consistently higher in one partner   The values ranged from 7·4 to 13 1 mg per cent   No bone changes were found histologically

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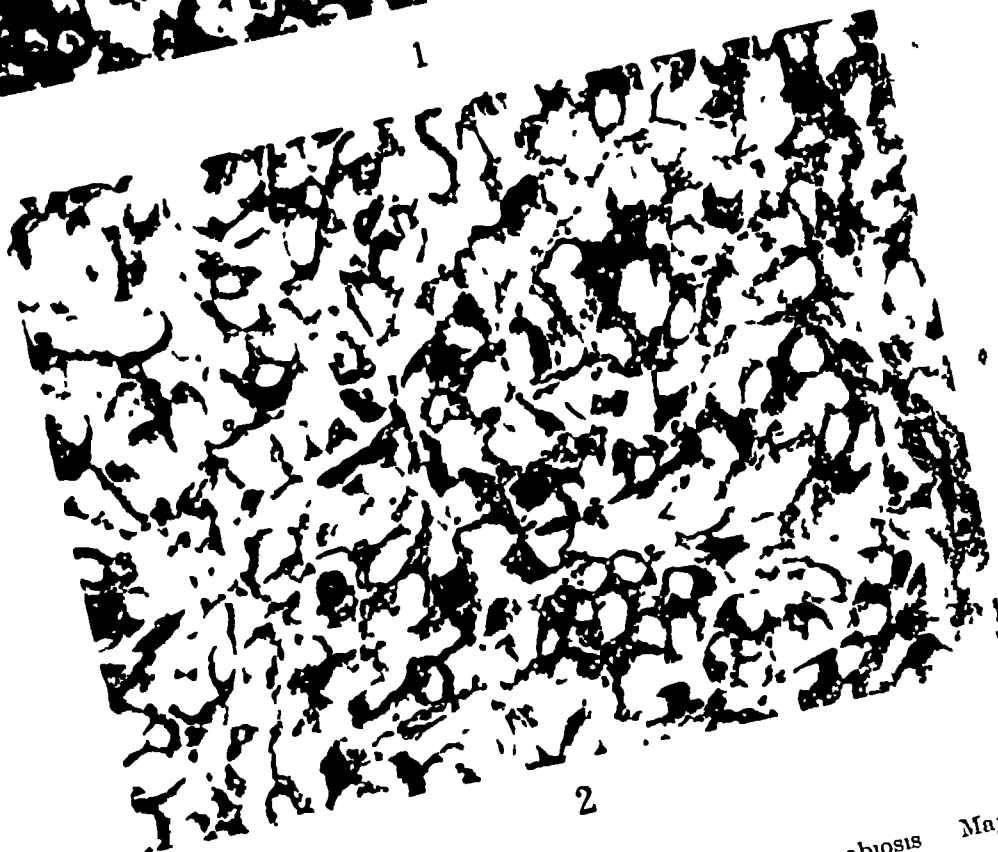
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Fig 1 —Parathyroid of intact partner (L) after 233 days of parabiosis   Majority of cells
represents the water clear type   × 650   H & E
Fig 2 —Parathyroid of normal rat of same age   × 650   H & E

Griesbach and Kennedy

which had not received any iodide supplement, was enlarged and showed hyperplastic epithelium   The basophilia (22 9 per cent) in this animal's pituitary is sufficient to account for the thyroid stimulation observed and is probably due to an iodine deficiency   The low thyroxine production in this pair was further evident from the small percentage of acidophil cells in the thyroidectomized partner's pituitary

Pairs No 2 to 5 had been given iodide for 4 months after thyroidectomy, but this had been withdrawn during the last 3½ months   The thyroid of pair No 5, while not enlarged, showed increased acinar cell heights and had apparently supplied the partner R with enough thyroxine to maintain a nearly normal pituitary   The remaining three pairs showed slight enlargement of the thyroids with activated epithelium   No conclusion can be drawn from these experiments as to the source of the thyrotropic hormone responsible for this slight activation The hormone may be derived from the animal's own pituitary or transferred from the thyroidectomized partner   This problem could be approached by parabiotic union of a thyroidectomized with a hypophysectomized animal Du Shane, Levine, Pfeiffer and Witschi (1935), who combined hypophysectomized with normal or castrated rats could not observe any thyrotropic effect on the hypophysectomy thyroid, and concluded that insufficient thyrotropin is transferred through the junction   The older experiments of Naiko and Ikonen (1934), and those presented in this paper, lead to the conclusion that even the large amounts of thyrotropin released by the thyroidectomy pituitary were not exerting any conspicuous action on the partner's thyroid

The enlargement of the parathyroids was quite striking   The cells appeared to be in a state of high activity, with a predominance of clear cells   This parathyroid enlargement and the cell changes were observed in all the intact animals Anselmino, Hoffmann and Herold (1934) described changes in rat parathyroids following injections of anterior pituitary extracts   The changes seen in our animals resembled closely those described by these authors, and suggested increased activity of the glands   This could be explained by the assumption that a parathyrotropic factor is transferred from the parathyroidectomized animal   The existence of a pituitary parathyrotropic hormone has been questioned, however, by other workers

SUMMARY

Thyroid-parathyroidectomy was carried out in one partner of parabiotically joined rats

The thyroids of the intact partners did not show any marked stimulation The amount of thyrotropic hormone passing from the thyroidectomized to the intact partner must therefore be negligible

Some thyroxine (equivalent to about 1 µg DL per 100 g per day) was transferred from the intact to the thyroidectomized partner

The parathyroids were enlarged and consisted predominantly of water clear cells

THE EFFECT OF SODIUM SALICYLATE UPON HYPERSENSITIVITY REACTIONS

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SALICYLATE treatment of rheumatic fever is based both on clinical observation and experimental work Its palliative effect is generally accepted, but, in addition, there is a considerable weight of evidence that it may arrest some of the pathological changes of the disease (Coburn, 1945, Reid, 1948, Small, Wissler and Watson, 1948) Whilst the etiology of rheumatic fever is still an unsolved problem there is little doubt that the vascular endothelium is involved from the earliest phases and that the lesions invite comparison with those resulting from antigen-antibody reactions of the hypersensitive or allergic type Indeed, much recent work supports the hypothesis that hypersensitivity, following upon infection with haemolytic streptococci, lies at the root of the rheumatic process Provisionally accepting this hypothesis it seemed to us that the effects of salicylate therapy might depend upon interference with hypersensitivity reactions and that, therefore, a study of the effects of salicylates on such reactions, experimentally induced in animals, might throw some light upon rheumatic fever pathogenesis During the course of the work Swyer (1948) published observations to the effect that in rabbits salicylates prevent the increased capillary permeability induced by intracutaneous injections of histamine We therefore extended our investigation to include experiments with certain anti-histamine drugs Swyer's claim was readily confirmed but the experiments revealed interesting differences between the actions of these anti-histamine drugs and of sodium salicylate The drugs used were " Antistin " (2-phenyl-benzylamino methyl imidazoline methane sulphonate), " Anthisan " (N-dimethylamino ethyl-N-*p*-methoxy-benzyl amino pyridine maleate), " Phenergan " (N-(2-dimethylamino-m-propyl) phenothiazine HCl) Throughout this paper they are referred to under their proprietary names

EXPERIMENTAL

Salicylate Treatment of Experimental Animals

Freshly prepared, Seitz filtered, aqueous solutions of sodium salicylate, B P standard, were used For intravenous and intraperitoneal administration the solutions were made approximately isotonic with 0 9 per cent NaCl (2 5 to 3 g per 100 ml) but for oral administration 5 per cent to 10 per cent solutions were used in order to reduce the volumes required

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* Attached to the department as a member of the external staff of the Medical Research Council

of recovery. From this record the degree of shock was assessed and the animal was given a shock score as follows:

Trivial symptoms	0 5
Mild shock	1
Severe shock	2
Very severe shock with eventual recovery	3
Death after more than 10 min	4
Death within 10 min	5

The results are summarized in Table II. They were closely similar with both actively and passively sensitized guinea-pigs. There was no clear-cut evidence of protection by salicylate treatment, but there was a tendency for treated animals to survive a little longer and to exhibit slightly less severe symptoms than controls. The difference appeared rather more striking during actual observation than the small score differences would suggest.

TABLE II.—*Effect of Sodium Salicylate on Anaphylactic Shock in Guinea-pigs*

Sensitization	Shock dose of antigen	Number of guinea-pigs		Average shock scores*	
		S	C	S	C
Active with Horse Serum ⎰ 0 01 ml intracardiac		10	11	3 5	4 3
⎱ 1–5 ml intraperitoneal		6	6	3 7	3 7
Passive with anti-bovine albumin rabbit serum	0 4–1 mg antibody N	6	6	3 4	4 1

S = Salicylate treated animals
C = Untreated controls
* Shock scores.—(For explanation see text.)

As a check upon the suitability of our experimental technique three actively sensitized guinea-pigs were each given 20 mg. per kg. Antistin intramuscularly and the shock dose of horse serum was inoculated intracardially 30 min. later. None of the animals showed any sign of acute anaphylaxis, but one to three hours later they were all mildly prostrated with ruffled fur. This supports the contention that although Antistin can suppress completely the immediate manifestations of anaphylactic shock, it may fail to prevent later prostration which is possibly caused by factors other than histamine (Rosenthal and Brown, 1940 ; Rose, 1947).

Arthus Reactions in Rabbits and Guinea-pigs

Effect of sodium salicylate upon direct Arthus reactions

An experiment was made in order to discover whether any clear effect of sodium salicylate could be demonstrated under the relatively uncontrollable conditions of the direct Arthus phenomenon in actively sensitized rabbits. Six animals were sensitized with human sera, and, on the basis of preliminary skin tests, were divided into two groups of as nearly equal sensitivity as possible.

TABLE III —*Effect of Sodium Salicylate on Reversed Passive Arthus Reactions in Rabbits*

Six rabbits in each group	Mean areas of oedema (cm²)		
	Group A	Group B	Combined A and B
When salicylate treated at 4 hr	3	2 3	2 7
,, ,, 24 ,,	3 7	3	3 4
When given NaCl as Controls at 4 hr	5 4	6	5 8
,, ,, 24 ,,	6 6	5 7	6 2

case, for in 11 out of the 12 animals the reactions during their period on salicylates were less at all stages of development than the reactions elicited during their period as control animals   The remaining animal was ill during the control period, which may have affected its skin reactivity

It was observed that small punctate haemorrhages occurred at the sites of the Arthus reactions in 10 out of the 12 rabbits when they were under salicylate treatment, but not when being treated with NaCl   We are unable to offer any explanation of this phenomenon

Guinea-pig experiment —A similar experiment was carried out with guinea-pigs   Two groups, each of five guinea-pigs, were used alternately as salicylate treated and untreated animals in duplicate tests   For salicylate treatment a total of 900 mg per kg was injected intraperitoneally in three doses at 30 min before, and 5 and 12 hours after, the intracutaneous inoculation of antibody, saline in corresponding volume being given to the untreated group   The antigen and antibody used were the same as in the rabbit experiment with dosage suitably adjusted for the smaller species   The antigen was given intracardially an hour before the antibody   Inoculation of the rabbit anti-serum into unsensitized guinea-pigs was shown to produce nothing more than a small, transient reaction   Guinea-pigs have seldom been used for experiments involving the Arthus phenomenon, probably because the skin reactions induced are less superficial and hence much less evident upon cursory examination than are the reactions in rabbits   It was indeed found impracticable to compare responses by measurement of surface areas alone, but valid comparisons could be based upon oedema volume calculated by the arbitrarily adopted formula, volume = ⅓ × surface area × depth   Five sets of measurements were made at intervals during the 24-hour period of observation   The results are presented graphically in the figure, mean oedema volumes for the ten animals when salicylate treated and when untreated being plotted against time   The wide separation of the two curves throughout the whole period of observation indicates the striking inhibitory effect of salicylate treatment upon the Arthus reaction in guinea-pigs

The effect of anti-histamine drugs on passive reversed Arthus reactions

The striking effect of Antistin in preventing acute anaphylactic shock in guinea-pigs led us to study the effect of this drug upon Arthus reactions in rabbits   The procedure was identical with that employed for the salicylate experiment except that the treated rabbits received three intramuscular injections of 50 mg Antistin during the first 12 hours, whilst control animals received no treatment
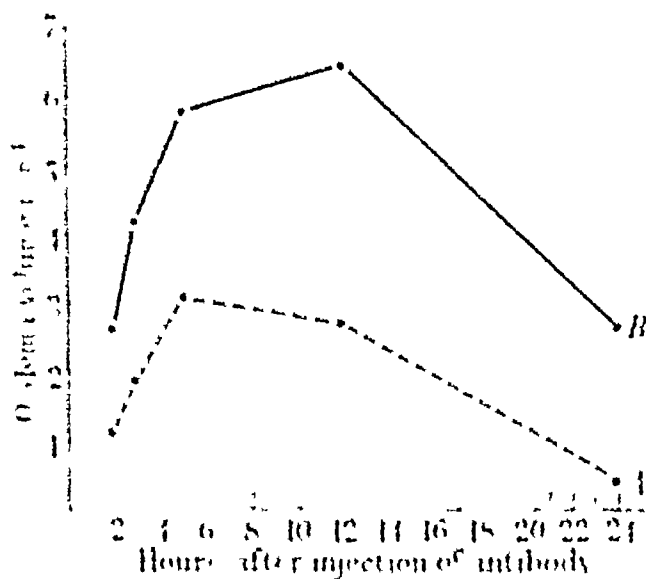
FIG. 1. Arthus reactions in 10 guinea-pigs

(A) Mean oedema volume of reactions induced during salicylate treatment
(B) Mean oedema volume of reactions induced during control period

at all. Ten rabbits were used, each group of five serving as controls to the other group in one of the two test. The results are given in Table IV. Reaction differences with and without Antistin are in the same direction as in the salicylate experiment but are very much less marked.

TABLE IV. *Effect of Antistin on Reversed Passive Arthus Reactions in Rabbits*

Five rabbits in each group	Mean volume of oedema (cm³)		
	Group A	Group B	Combined A and B
When Antistin treated at 4–5 hr	5.6	5.8	5.7
,, ,, 24 ,,	7.8	7.5	7.7
When untreated controls at 4.5 hr	5.9	8.9	7.4
,, , 24 ,,	7.8	8.9	8.3

Both Antistin and Anthisan were also tested in guinea-pig experiments on groups of 8 and 6 animals respectively. In each case the relatively very large amount of 60 mg of the drug was given intramuscularly in three equal doses during the observation period. When mean oedema volumes were plotted the curves for Antistin, though separate, were much closer together than those shown in the salicylate experiment (Fig 1), whilst the curves for Anthisan were practically superimposed. Thus, the result of the rabbit test of Antistin was confirmed and Anthisan was shown to have practically no effect at all upon Arthus reactions in guinea-pigs.

These results are surprising in view of the efficacy of Antistin and the inefficacy of sodium salicylates in the acute anaphylaxis experiments recorded above and they indicate that the action of sodium salicylate is not just a direct

anti-histamine effect   Furthermore they suggest that factors, other than histamine, are important in the development of the Arthus phenomenon

The Shwartzman Reaction

Effect of Sodium Salicylate

The Shwartzman phenomenon was produced in rabbits as described by Shwartzman (1938)   Berkefeld N filtrates were prepared from agar cultures of two strains of *Bact coli* from the National Collection of Type Cultures Injections of 0 25 ml of *Bact coli* 86 filtrate were made at two or three separate sites into the shaved abdominal skin and 24 hours later a dose of 0·1 ml per kg to 0 5 ml per kg of the less toxic *Bact coli* 7020 filtrate was injected intravenously   Any areas of haemorrhage and oedema which developed were measured at 5 hours and 24 hours   In some experiments the treated animals received intraperitoneal injections of 3 per cent sodium salicylate at a dosage of 0 4 g per kg 15 min before and 7½ hours after the intracutaneous injections, and a further two doses half hour before and 6½ hours after the intravenous injection   In other experiments the last two doses were omitted, salicylate being administered during the skin preparatory period only   Control animals received no treatment

The results of seven such experiments are presented in Table V   Whilst there were no significant differences between treated and untreated groups as

TABLE V —*Effect of Sodium Salicylate on the Shwartzman Reaction*

Experiment	Number of rabbits	Treatment	Areas of haemorrhage (cm²)			
1	2	Salicylates in Stages 1 and 2	0	0		
	2	Controls	0 64	1 75		
2	2	Salicylates in Stages 1 and 2	0 88	1 1		
	4	Controls	3 1	3 1	1 86	1 7
3	2	Salicylates in Stage 1	0	0		
	2	Controls	1 1	0 4		
4*	5	Salicylates in Stage 1	0	0	0	0
	5	Controls	0 4	0 1	0	0
5	3	Salicylates in Stage 1	0	0	0 1	
	3	Controls	2 8	0 8	0 8	
6	2	Salicylates in Stage 2	0	0 2		
	2	Controls	0 1	1 5		
7	2	Salicylates in Stage 2	0	1 2		
	2	Controls	0 6	3 1		

* Filtrate used in Experiment 4 was of very low potency owing to storage at 4° C

ic and the degree of prostration following the intravenous inoculations the effect of salicylate treatment was strikingly manifest in the severity of the haemorrhagic skin lesions. In all experiments in which treated animals showed any reactions at all these were trivial compared to those in the controls and in three experiments the salicylate entirely suppressed their development. Moreover it is clear that it is not necessary to give the drug throughout both stages. Given in Stage I only it appears to be every bit as effective as when given in both.

The effect of Anthisan

Four rabbits were given intramuscular injections of Anthisan 50 mg at 5 min before the skin preparatory injections, 50 mg 5 hours after and 25 mg 18 hours after. Two of the animals were given a further injection of 50 mg 5 min before the intravenous inoculation of *Bart coli* filtrate. Two control animals received no treatment.

In contrast to the salicylate experiments no appreciable differences between treated and control rabbits could be detected in respect of the areas of haemorrhage which are the essential feature of the Shwartzman reaction although oedema when it occurred was less in the treated group. The whole group reacted with such remarkable uniformity that mean areas of haemorrhage were identical in five rabbits and only slightly larger in the sixth, which was one of the treated animals. These results, therefore confirm the conclusion drawn from the Arthus experiments that the focus of action of sodium salicylate is different from that of the histamine antagonists.

The Mode of Action of Salicylates

Inhibition of histamine lesions

The importance of histamine release as a factor in the development of hypersensitivity reactions suggested the study of the effects of salicylate treatment on lesions produced by intracutaneous inoculations of histamine. It is unnecessary to record the experiments in detail because whilst the work was in progress a report of the anti histamine effect of sodium salicylate was published by Swyer (1948) and our own work fully confirms his claim that administration of sodium salicylate inhibits the increased capillary permeability associated with histamine skin lesions. In early experiments we used intravenous injection of 5 per cent trypan blue given 5 to 10 min before intracutaneous inoculation of freshly-diluted histamine acid phosphate and the rabbits were divided into salicylate treated and untreated control groups the drug being administered intraperitoneally. Later we repeated Swyer's experiments more exactly by using pontamine blue as the vital dye and using each rabbit as its own control by testing it both before and after sodium salicylate had been given either intraperitoneally or intravenously. Plasma salicylate levels were obtained on specimens obtained just before the histamine inoculations. It was shown in preliminary experiments that the necessary interval of 30 to 45 min between first and second tests on the same animal did not in itself influence the extent of " blueing " at histamine inoculation sites. All the experiments showed definite reduction of extravasation of dye when the blood plasma contained an adequate concentration of salicylate

The possibility that this action of sodium salicylate might depend upon the formation of an inactive salicylate-histamine complex was investigated by testing a series of salicylate-histamine mixtures These were made in isotonic phosphate buffer solution pH 7 5 and contained, per 0 2 ml volume, 0 005 mg histamine acid phosphate with 1, 0 33, 0 11, 0 03, 0 01 and 0 mg sodium salicylate Each of four rabbits received intracutaneous inoculations of each mixture, and also of similar doses of salicylate without histamine after an intravenous inoculation of pontamine blue solution The results were extremely uniform, readings at 15, 30 and 120 min showing blueing at all histamine sites without any appreciable effect of sodium salicylate at any dose The inoculations of the salicylate doses without histamine caused no blueing at all

Effect on capillary permeability in the Arthus and Shwartzman reactions

The vital dye technique was used to study the effects of sodium salicylate and Anthisan upon capillary permeability at Arthus reaction sites in rabbits Reversed passive Arthus reactions were produced as in previous experiments except that a smaller dose of antibody (0 12 mg antibody N) was used for the intracutaneous injections Treated rabbits were given sodium salicylate 400 mg per kg intraperitoneally or Anthisan 20 mg per kg intramuscularly at the same time as the sensitizing intravenous inoculation of antigen Additional control animals received skin inoculations of antibody but no antigen The vital dye was injected intravenously 15 min before the skin inoculations of antibody in the form of 5 per cent solution of pontamine blue, 0 7 ml per kg dose Estimates of intensity and measurements of areas of blueing were made at intervals

The results are given in Table VI Anthisan had no appreciable effect although adequacy of dose was proved by complete inhibition of blueing at sites inoculated with 0 01 mg histamine acid phosphate in the same animals This makes all the more interesting the striking inhibition by salicylate treatment In these salicylate rabbits the extravasation of dye was no more than occurred in the additional controls as a result of inoculation trauma

TABLE VI —*The Effect of Sodium Salicylate and of Anthisan on Capillary Permeability During the Arthus Reaction*

Number of rabbits	Drug treatment	Degree of " blueing " at skin moculation sites					
4	Sodium salicylate, 400 mg per kg	+	+	±	±		
2	Anthisan, 20 mg per kg	+++	+++				
6	*Nil*	+++	+++	+++	+++	+++	+++
5	*Nil**	+	+	+	±	±	

* These additional controls received no antigen intravenously , hence no Arthus reactions occurred at sites of antibody injections The slight blueing is attributable to inoculation trauma

A preliminary experiment was carried out with another anti-histamine drug, Phenergan, because of a recent report that it is capable of suppressing the development of the Shwartzman phenomenon in rabbits (Vallery-Radot, Halpern and Reber, 1948) Phenergan is a synthetic compound derived from pheno-

inoculated locally, was used to provoke increased permeability of the capillaries of the skin. Swyer's results were readily confirmed by us, but our experiments lend no support to his suggestions that salicylates have anti-histamine properties and that their anti-rheumatic and anti-histamine actions may be causally related. It is more probable that sodium salicylate directly affects the walls of the capillaries though the mode of action remains obscure.

If this is the case the contrasting behaviours of sodium salicylate and the anti-histamine drugs in the various types of experiment recorded become explicable. Acute anaphylactic shock in the guinea-pig is generally considered to be due to histamine release at tissue sites of great histamine sensitivity. It is thus not surprising that anti-histamine drugs can afford complete protection against immediate shock although the protected animals may show ill effects some time later, suggesting that factors other than histamine are also involved in the reaction. Similar protection is not to be expected from sodium salicylate which is apparently capable of stabilizing the threshold of capillary permeability but which, so far as is known, has no direct neutralizing action on histamine. In reactions of the Arthus type and in the Shwartzman phenomenon the position is reversed; sodium salicylate exerts a protective action whilst the anti-histamine drugs are impotent. The part played by histamine in such local hypersensitivity reactions is obscure. The slow development of erythema, oedema and necrosis over a period of several hours contrasts with both the dramatic suddenness of anaphylactic shock and the rapid development and rapid disappearance of the local reactions produced by single intracutaneous injections of histamine. The tissue toxin responsible, whatever its nature may be, appears to be either formed or released at the reaction site continuously over a prolonged period and the mechanism involves transport of reagents like antigens or antibodies to the site via the blood stream. In such circumstances capillary permeability becomes a most important factor, and the fact that lowered permeability thresholds at the reaction sites are an integral part of the Arthus and Shwartzman reactions is easy to demonstrate directly by the *intra-vitam* dye technique. Just how and when the capillary change is induced remains unknown, though in the Shwartzman reaction it appears to occur during the first, or skin preparatory stage as a result of the local injection of bacterial filtrate; hence in this latter reaction a high blood concentration of sodium salicylate during the first stage only is almost as effective as when it is maintained over the whole period of reaction.

It is not possible to assess at the present time the significance of these results in the major problem of the pathogenesis of rheumatic fever. They throw no light on the question of etiology, but if local hypersensitivity reactions caused by the interaction of streptococci, or streptococcal-tissue complexes, or auto-antigens, with homologous antibodies are at the root of some of the pathological changes which characterize the disease then the palliative effects of salicylate therapy become readily explicable on the basis of the stabilization of capillary permeability. Furthermore, the necessity for long continued dosage is obvious. It is, of course, not suggested that this action of salicylates is their only pharmacological effect. Other actions, such as the compensated alkalosis due to the induced hyperpnoea demonstrated by Reid (1948), may also play a part although in our experiments we were unable to detect any significant change in the alkali reserve of the plasma of rabbits maintained on sodium salicylate throughout the periods of observation.

SUMMARY

Sodium salicylate in blood concentrations of the order of 50 mg per 100 ml confers no appreciable protection against acute anaphylactic shock in guinea-pigs With the same technique of shock production the anti-histamine drug, Antistin, was found to protect completely against the immediate fatal effects

In passive Arthus reactions in both guinea-pigs and rabbits, sodium salicylate exerts a protective action whilst the anti-histamine drugs are relatively impotent The same is true to an even greater extent in the case of the Shwartzman phenomenon

This protective effect of sodium salicylate is not a direct anti-histamine effect but appears to be due to some action on the local blood capillaries The increased capillary permeability which normally occurs in such hypersensitivity reactions is thus prevented

The implications of these results in respect of rheumatic fever are briefly discussed

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AN INVESTIGATION INTO THE EFFECT OF COMBINED DIPHTHERIA-TETANUS-PERTUSSIS PROPHYLACTIC [*]

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WHEN different antigens are combined to give a polyvalent mixture, and the mixture intended for human prophylaxis, it is of particular importance to ascertain the effect of the presence of the others on the antigenicity of a given one

It has been established that when some combinations of antigens are used, the response to one may suffer appreciably, compared with its activity as an isolated antigen   Michaelis (1904) called this phenomenon "The competition of antigens," and his opinion has been confirmed by Glenny and Waddington (1926), Hektoen and Delves (1932) and others   On the other hand, Tomcsik (1939), while studying this phenomenon, stressed the importance of the time factor, since the individual antibody responses from a combined preparation, may reach their maximal values at different times, and the duration of the immunity produced may also be different   In view, therefore of the different responses, the best time to give the second inoculations may be difficult to judge accurately

Reactions provoked by combined antigens constitute an entirely separate problem , we have shown, in respect of combined diphtheria-scarlet fever prophylactic, that there is a cumulative effect on repeated inoculation (Farago, 1938)

This communication is concerned with the results of studies made in respect of the first two problems only

METHODS

The diphtheria toxoid used in these experiments was prepared by the method of Holt (1948) , the purity of this material was 1,100 Lf/mg N

The *H pertussis* vaccine was produced in the semi-synthetic medium of Cohen and Wheeler (1946) modified to give a solid medium by the addition of 2 5 per cent agar   The microbes derived from cultures on this medium were proved to be in Phase I

The tetanus toxoid was prepared from culture in the semi-synthetic medium of Mueller and Miller (1941)   The crude tetanus toxoid contained the equivalent of 3300 guinea-pig M L D /ml of toxin   This was purified by the use of charcoal, and repeated adsorption onto $Al(OH)_3$ and dialysis   The final product had a purity of 39,000 eq M L D /mg N

For these experiments we used two preparations   (i) A suspension of *H pertussis* in a solution of tetanus and diphtheria toxoid, and (ii) an *adsorbed* combined prophylactic using Holt's $AlPO_4$ (1947) as the mineral carrier , this at a concentration of 7 mg $AlPO_4$ ml

For *controls* we used the three components of the combined prophylactic separately, each with and without the addition of the mineral carrier   It should be recorded that all these preparations were preserved by the addition of 1/10,000 sodium ethyl mercuri thiosalicylate, and passed the usual sterility and innocuity tests

Composition of the Reagents Employed

(per ml)

Type		Combined	Isolated
" Fluid " (1)	{	10 Lf diphtheria toxoid 20 10⁹ *H pertussis* 100 cq MDL tetanus toxoid (2 Lf U)	(2) 10 Lf diphtheria toxoid (3) 20 10⁹ *H pertussis* (4) 100 cq MLD tetanus toxoid (2 Lf U)
Adsorbed (5)	{	10 Lf diphtheria toxoid 20 10⁹ *H pertussis* 100 cq MLD tetanus toxoid, 7 mg AlPO₄	(6) 10 Lf diphtheria toxoid with 7 mg AlPO₄ (7) 20 10⁹ *H pertussis* with 7 mg AlPO₄ (8) 100 cq MLD tetanus toxoid 7 mg AlPO₄

Groups of five chinchilla rabbits of the same breed, over 3 kg in weight, were inoculated subcutaneously with 1 ml of each of the eight preparations detailed above , the second injections were also 1 ml

The antibody responses were determined at short intervals after inoculation, by taking ear-vein bleedings, and making accurately pooled samples of the sera from each group of animals   The diptheria antitoxin titrations were made using the method of Jensen (1933) against a standard antitoxin kindly supplied by the Statens Seruminstitut, Kobenhavn   Tetanus antitoxin titrations were made using a slight modification of Istratis (1938) micromethod, and *H pertussis* agglutination titres with a heat-killed standard suspension of *H pertussis*, also checked against a standard immune-serum

RESULTS

The results obtained from each preparation are shown graphically in Fig 1–8

1   *In respect of diphtheria toxoid*, we find that the responses to plain toxoid (non-adsorbed or mixed with vaccine) was the production of very small amounts of antitoxin, and this only reached a useful level after the third injection (Fig 4)   The same amount of toxoid adsorbed onto 7 mg of AlPO₄ gave a greatly superior antitoxin response, in that even 21 days after one injection the serum titre was as high as after the third using plain toxoid, and the titre was still rising in respect of the adsorbed toxoid (Fig 3)   Even so, the best antitoxin responses were obtained after two or three doses of the combined prophylactic together with AlPO₄ (Fig 1)

2   *In respect of tetanus toxoid*, the results obtained were, by and large, similar to that using diphtheria toxoid , using plain toxoid, a poor response followed the first dose, and sluggish responses to the second and third (Fig 8)   Combination of tetanus toxoid with diphtheria toxoid and *H pertussis* vaccine gave much better results (Fig 2), and also by adsorption onto AlPO₄ (Fig 7), and the best results using the combined prophylactic with mineral carrier (Fig 1)   It may be pointed out here that the recovery from the negative phase following
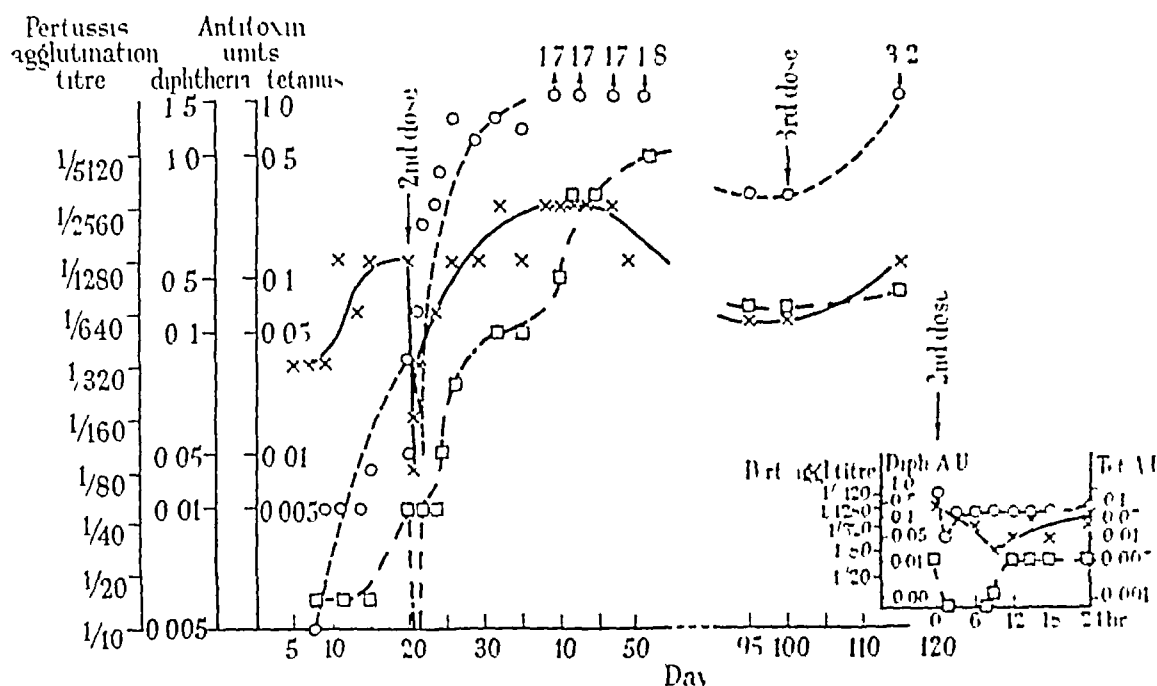
Fig 1 —The combined diphtheria pertussis tetanus vaccine adsorbed to AlPO₄

O——O Diphtheria   □ — □ Tetanus   ×——— Pertussis
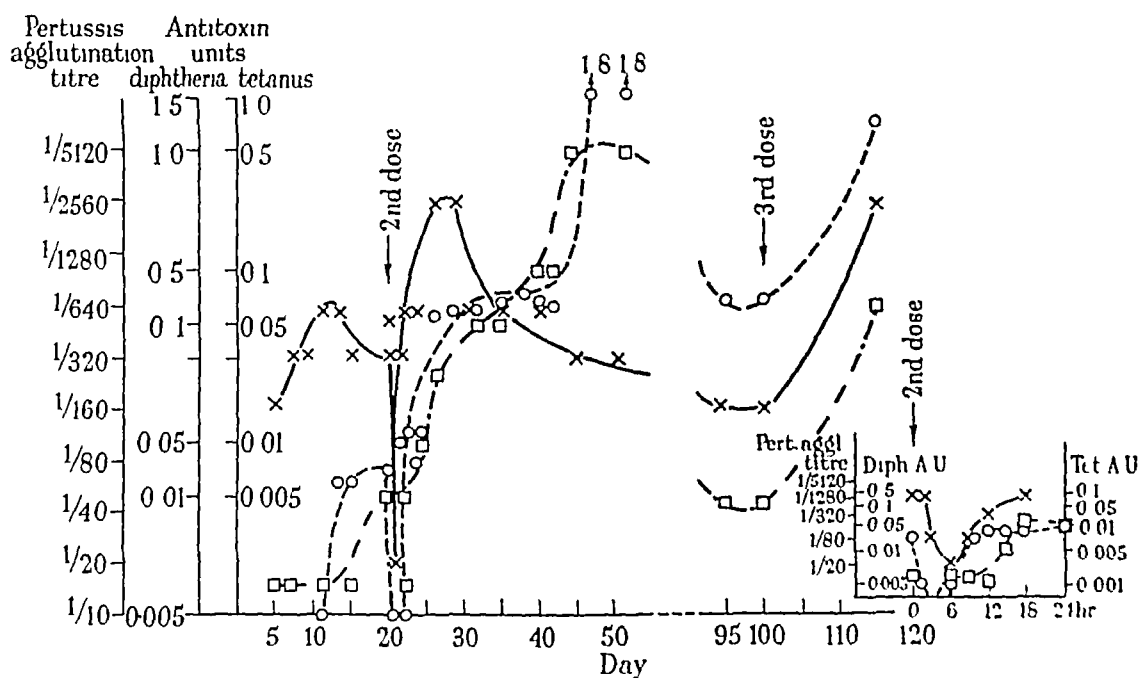
Fig 2 —The combined diphtheria pertussis tetanus vaccine

O——O Diphtheria   □ — □ Tetanus   ×——— × Pertussis
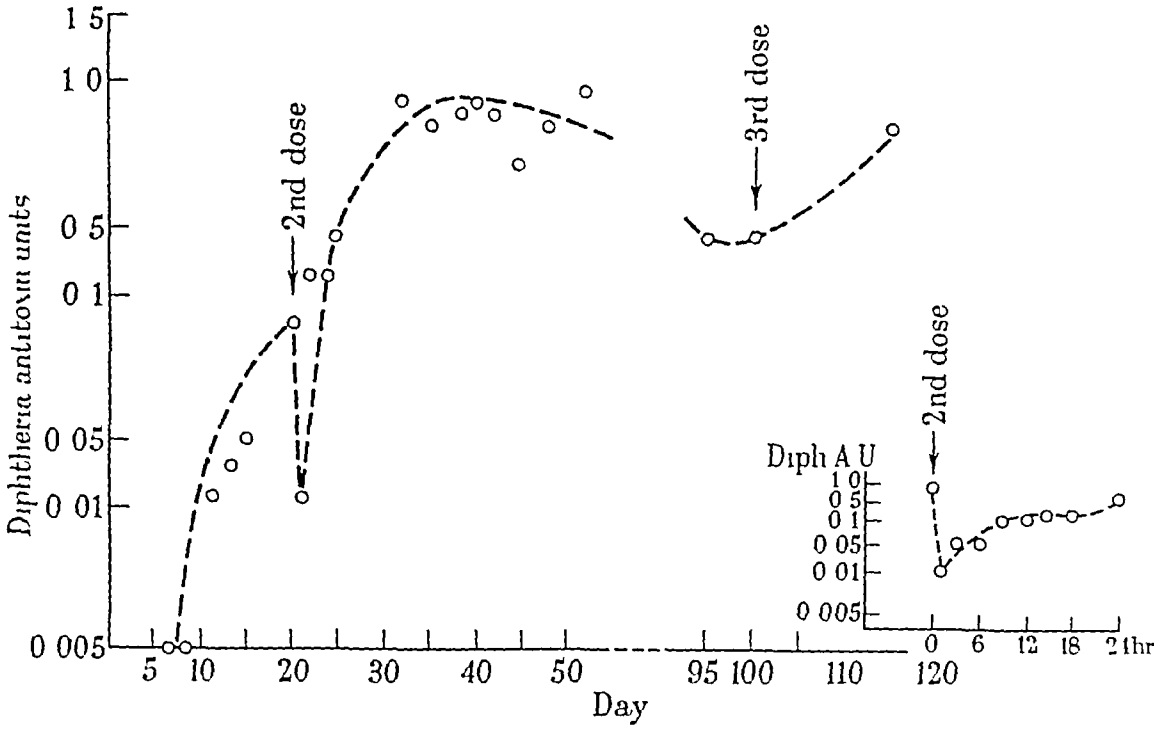
Fig 3 —Diphtheria toxoid purified and adsorbed to AlPO₄
O — — O Diphtheria
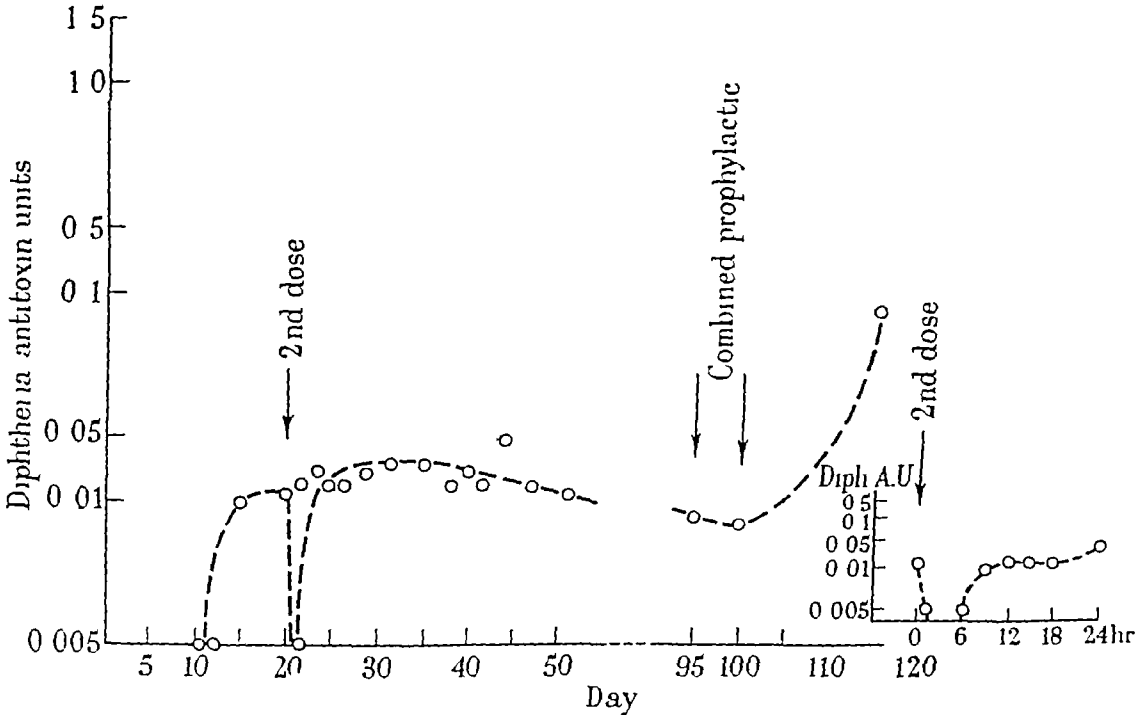
Fig 4.—Diphtheria toxoid purified
O — — O Diphtheria
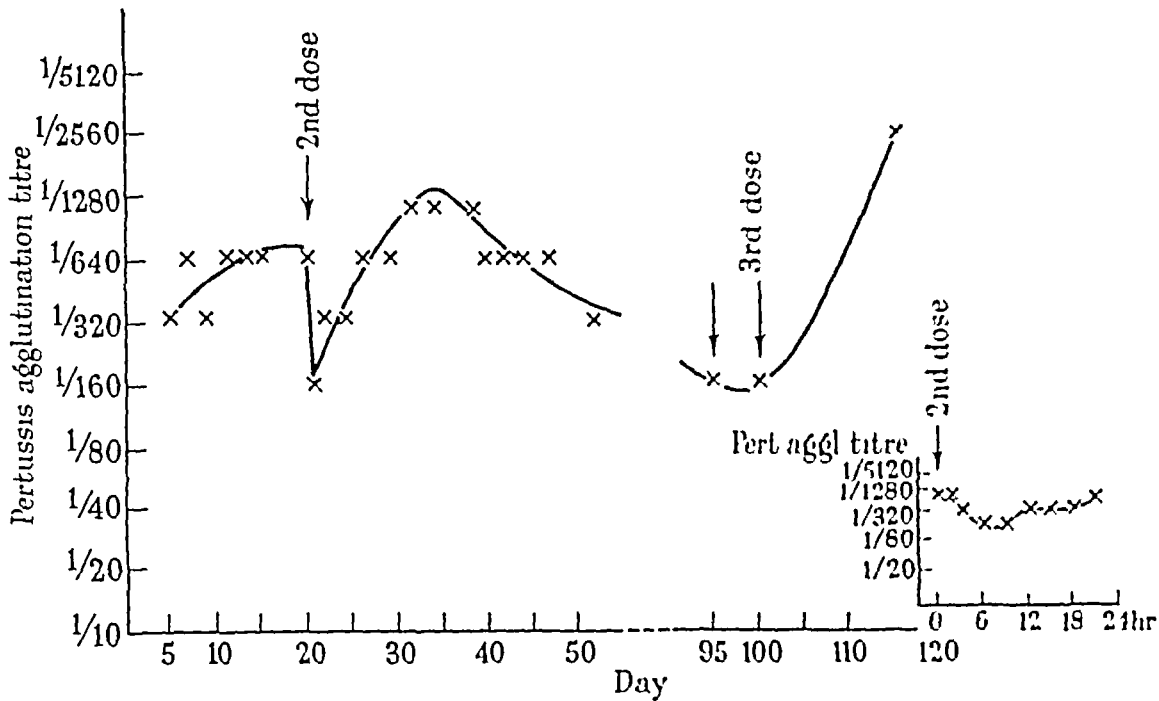
FIG 5 —Pertussis vaccine adsorbed to AlPO₄
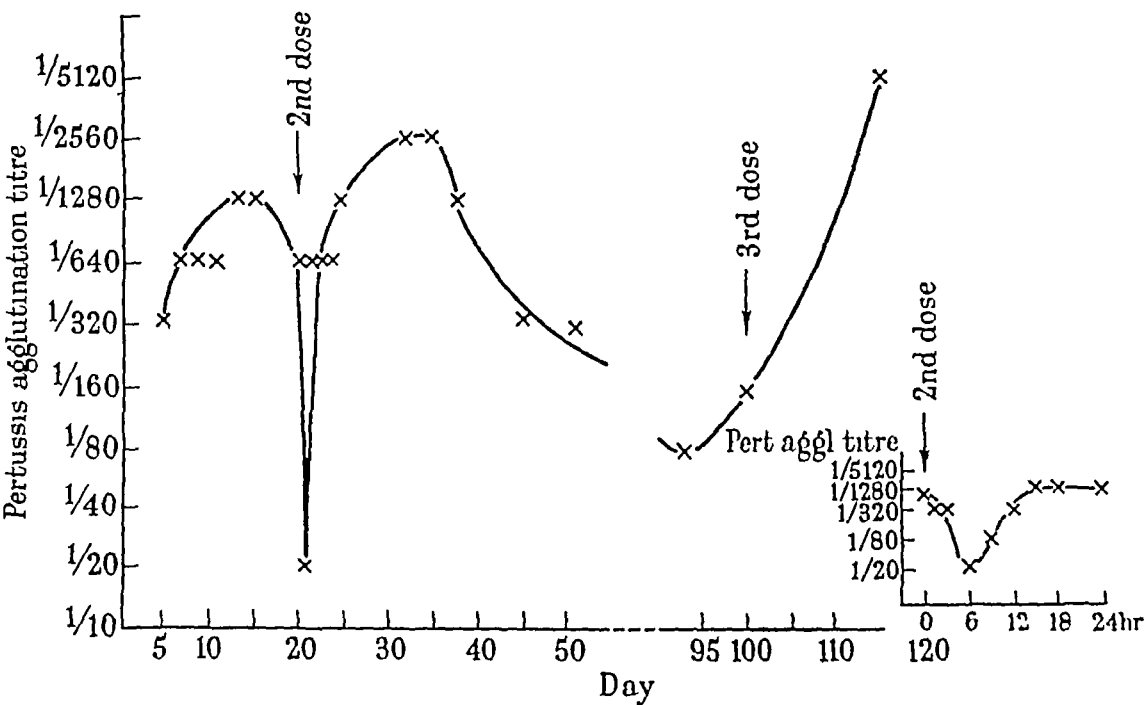
×———— / Pertussis

FIG 6 —Pertussis vaccine

×———— × Pertussis
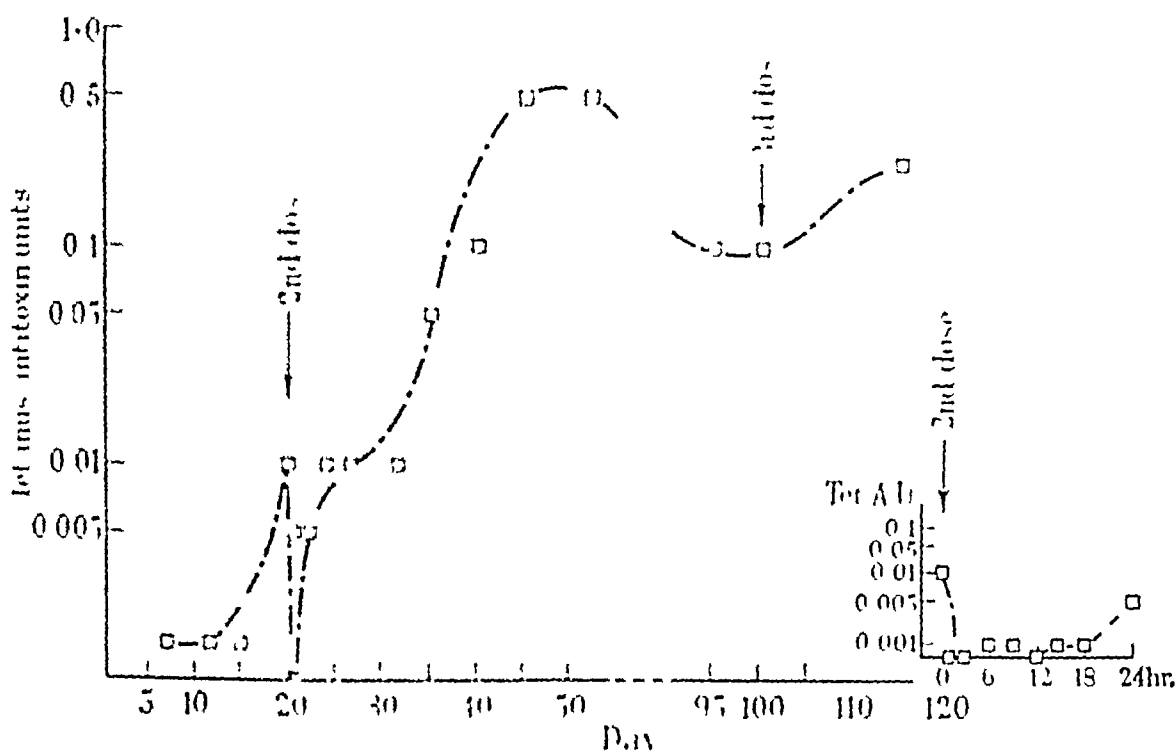
Fig 7.—Tetanus toxoid purified and adsorbed to $AlPO_4$
□ — □ Tetanus
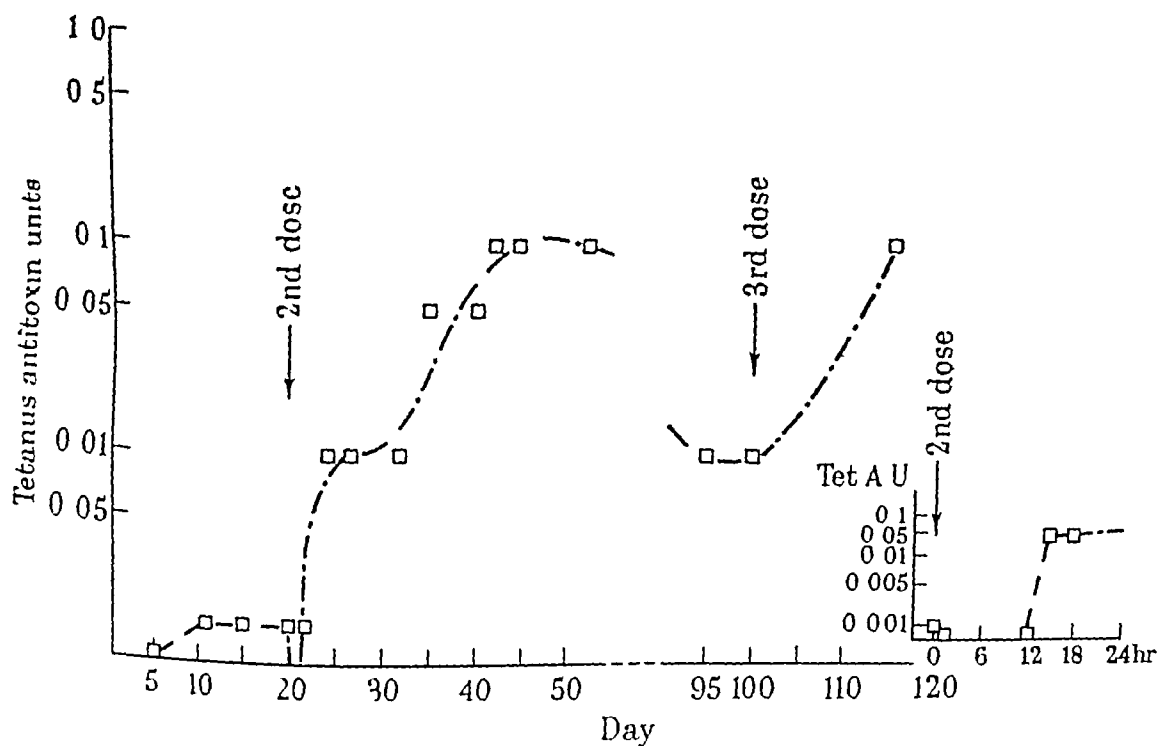
Fig 8.—Tetanus toxoid purified
□ — □ Tetanus

the second injection was not as steep as with diphtheria antitoxin, but showed a marked lag before ascending to its peak value   This phenomenon was definite in respect of all three components using the combined preparations, and particularly noticeable in respect of tetanus antitoxin responses, and is probably a phenomenon directly referable to the negative phase

3   *In respect of H pertussis,* no marked differences were obtained   After the second and third inoculations of the plain vaccine there occurred a rapid rise and decrease in agglutination titre (Fig 6)   In respect of maximum titres, no significant differences were observed between the results from either the combined prophylactic (Fig 1 and 2) or with mineral carrier (Fig 5), all values were comparatively uniform   Nevertheless, it will be noticed that the response to the vaccine with $AlPO_4$ carrier, was definitely flatter, which suggests that the immunity induced was slower to become established, and slower to recede (Fig 1 and 5) , alternatively, the duration of antigenic stimulation may have been prolonged by association with the carrier

Some points of interest also arise with regard to the negative phase   Details of this phenomenon are shown in the bottom right-hand corner of Fig 1–5 where the time scale is in hours   Observations were made one hour before the second injection, 1, 3, 6, 9, 12, 16 and 18 hours afterwards   In every instance was there a fall in circulating antibody corresponding to the antigen injected, although less in degree, but more protracted in the case of antigens with mineral carrier This latter may be due to the greater amount of antibody present in the animals before injection, and/or to a less ready fixation of the antigen by host antibody The most marked decline in titre followed the second injection of tetanus toxoid, and this persisted for as long as twelve hours, less pronounced in respect of diphtheria antitoxin, and only slight in the case of pertussis agglutinins

Certain considerations led us to determine to what extent, if any, the injection of non-specific substances had a stimulating effect on pre-existing antibody responses

For this we employed two rabbits from each group , 75 days after their second inoculations, they were injected as follows

(i) Those which received the $AlPO_4$ carrier with their former injections were given a dose of *E typhi*

(iia) Those which received plain tetanus or diphtheria toxoid, a dose of *H pertussis*, and

(iib) Those which received *H pertussis* vaccine alone, or tetanus toxoid, a dose of plain diphtheria toxoid

Neither the non-specific, nor the non-related tertiary inocula caused any increase in the animals' pre-existing antibody titres, except, to a small degree, following the injection of typhoid vaccine   These results confirm our former observations (Faragó and Ujhelyi, 1942)

DISCUSSION

From the point of view of the primary objective of this work, namely to determine if one or other of the three antigens used depressed the antigenicity of another when injected as a mixture, we have not observed any " competition of antigen " phenomenon   On the contrary, we have clear-cut evidence for

stating that the blending of *H pertussis* vaccine with plain diphtheria and tetanus toxoids, increases markedly the antitoxin responses to the latter antigens

Placing our findings in order of merit, the best results were obtained with the combined antigens associated with the $AlPO_4$ carrier, followed by the simple mixture, and the isolated toxoids a very poor third   In respect of the responses, to the pertussis vaccine, it is to be noted that the agglutination titres obtained were substantially the same, no matter how the vaccine was inoculated

As yet we do not know precisely the mechanism of the potency augmenting effect of the vaccine (pertussis) on toxoid antigens   It may possibly belong to the intricate pattern of depôt formation described by Faragó (1935), Holt (1947 , 1949), and Holt and Bousfield (1949) , on the other hand it may be due to other causes   We are at present investigating the latter possibility, and the results to date support the conclusions of Wagner-Sakharova (1937, 1938), Schutze (1940), Sauer and Tucker (1942) and Kendrick (1943)   Ordmann and Grasset (1945) have recently described good results from a combined diphtheria-pertussis prophylactic using it for both human immunization and animal tests

In respect of tetanus antitoxin production, in so far as the above experiments are referable to human prophylaxis, they would indicate that three spaced inoculations are advisable to ensure satisfactory immunization   The use of rabbits is perhaps to employ an animal that does not flatter antitoxin responses Maclean and Holt (1940), studying human material, found two doses of formol-toxoid without the addition of typhoid vaccine resulted in good immunity, and that the addition of the vaccine gave a five-fold increase in antitoxin response Nevertheless, we recommend a greater quantity of tetanus toxoid in the combined prophylactic for human immunization

In reference to the time factor in the responses to these antigens, as pointed out by Tomcsik, there is a very definite difference in the rates of production of diphtheria and tetanus antitoxins, and in agglutinins to *H pertussis*

These and other differences are most marked after the second inoculations In respect of pertussis agglutinins, the time response curve of serum titres was always smooth , they reached their maximum value after the second injection when combined with tetanus and diphtheria toxoids, some fifteen days when alone or associated with the $AlPO_4$ carrier, and twenty days when combined and associated with $AlPO_4$ The antitoxin response to diphtheria toxoid alone was as usual, giving a peak value ten days after the second injection, and some 17 days when adsorbed onto $AlPO_4$, but when combined with pertussis vaccine and tetanus toxoid showed a curious two-stage response, one reaching a maximum some 15 days after the second inoculation, and a second, some four times greater in amount, after 30 days with a flat peak   Using the combined prophylactic associated with $AlPO_4$, the response was continuously smooth to give a maximum value at about the 20th day with a very flat peak, 12 days or more   In respect of the tetanus antitoxin responses, except perhaps in the case of plain toxoid, a two-stage response was obtained in every case, at approximately the 10th and 25th day after the second injection respectively   The significance of this phenomenon is obscure, but clearly one of importance when assessing potency, and in no way detracts from the value of such a combined prophylactic for human immunization

Finally, our experiments dealt with the negative phase as one likely to be of some importance in practical immunization   The question seemed to be,

would the simultaneous inoculation of three antigens result in an exaggerated or prolonged negative phase ? A study of the figures detailing our results show that there is no essential difference using combined or isolated antigens, except that in the cases of the combined antigens plus AlPO₄, since the initial antibody titres were relatively high, the lowest titres obtained followed the use of isolated antigens Madsen and Jensen (1943) who also used rabbits, analysed carefully the negative phase in respect of diphtheria antitoxin and found it required three days for the height of the initial titre to return following injections of diphtheria toxin This return to normal during the latent period for the second response phenomenon, would be accounted for by Glenny and Barr (1949) and Holt s contention (1949) that antitoxin is being continuously produced in an immunized animal We are of the opinion that the less pronounced negative phase in our experiments, represent a state of affairs nearer to that obtaining in human immunization than that observed by Madsen and Jensen who used large amounts of toxin

SUMMARY

1 The comparative antigenicity of tetanus and diphtheria toxoids and *H pertussis* vaccine were observed in combination and also adsorbed onto AlPO₄ gel, with the same antigens used alone, and adsorbed separately onto AlPO₄ Groups of five rabbits were used for each of the eight preparations, and the antibody titres determined on pooled sera

2 The most effective preparation was the combined mixture with AlPO₄ carrier, followed by the combined mixture without mineral carrier The simple unadsorbed toxoids gave very poor responses

3 Although the presence of *H pertussis* vaccine in the combined prophylactic resulted in a greatly enhanced antitoxin response from both the tetanus and diphtheria toxoids, there was no augmentation in the pertussis agglutinin titre from any mixture

4 As the responses to primary, secondary and tertiary stimuli were closely followed, the different speeds at which antitoxic and antibacterial titres developed in the blood did not cause confusion in our reading of the results Timing differences due to the use of AlPO₄ were thereby also accurately observed

5 An apparently new and as yet unexplained phenomenon is described— a two-stage second response to tetanus toxoid, and under certain circumstances to diphtheria toxoid

6 The negative phase, following reinoculation, was also measured The immediate temporary fall in circulating antibody was most marked in the cases of plain toxoid, and less so with the AlPO₄ adsorbed toxoids There was no exaggeration of the negative phase as a result of blending the three antigens

7 The findings are discussed in relation to their possible mechanism and to human immunization

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TISSUE NEUTRALIZATION OF LOUPING-ILL VIRUS

D G ff EDWARD

From the Wellcome Veterinary Research Station Frant, Sussex

In an investigation of louping-ill in sheep, which had been inoculated subcutaneously with virus, it was frequently impossible to demonstrate virus in the brain after the animal had succumbed to encephalitis In view of the observation of Gard (1944) that the brains of mice infected with poliomyelitis contained a neutralizing substance which appeared to be distinct from anti-body, an examination has been made of the brains of sheep infected with louping-ill to ascertain whether failure to isolate virus was dependent on the presence of a similar neutralizing substance

Examination of brains for demonstrable virus

The brains of 30 sheep, in which fatal encephalitis had been induced by subcutaneous inoculation with louping-ill virus, followed three days later by an intracerebral injection of starch (Edward, 1947), were tested for virus shortly after the animals had died or had been killed when moribund In only 19 (63 per cent) of the brains could virus be demonstrated by the intracerebral inoculation of mice In 13 of these virus was plentiful, there being a 100 per cent mortality among the mice inoculated with a 10 per cent suspension Titration of a few of the brain suspensions showed that the LD_{50} titre reached $10^{-4 \cdot 8}$ Virus was apparently scanty in 6 brains because only a few of the inoculated mice died In the other 11 (37 per cent) brains no virus could be demonstrated

Among the strongly positive brains the earliest time at which the material was examined was the sixth day after inoculation with virus, and the latest was the ninth day Two brains obtained on the thirteenth and fifteenth days were weakly positive On the other hand, all but two of the brains found to be negative came from animals which succumbed between the tenth and sixteenth days The exceptions were brains from two sheep in one experiment which showed nervous signs four days after inoculation and were seriously paralysed the next day It is exceptional for louping-ill encephalitis to develop as soon as this, and, although the paralysis was typical, it is open to doubt whether the deaths were specific

Of the sheep whose brains were examined, 6 had never been vaccinated against louping-ill , virus was present in all except one of these brains The one negative brain was examined 14 days after inoculation, the animal having remained in a paralysed condition for 4 days The other brains were obtained in challenge experiments from sheep which had succumbed in spite of attempts to protect them with formalinized vaccines A few negative brains came from

animals vaccinated with materials whose inability to protect any of the animals inoculated suggested that they contained little or no specific antigen

All except two of the brains found to contain virus were from sheep which had succumbed 24 hours or less after the first signs of encephalitis had been noticed   A higher proportion of those brains with no demonstrable virus came from animals which had persisted longer in a paralysed state   No virus, however, could be found in 6 brains even though they were examined 24 hours or less after encephalitis was first detected

Neutralizing substance in infected brains

In order to find out if the failure to demonstrate virus was due to the presence of a neutralizing substance, suspensions of the brains (10 per cent) were made, using as diluent M/50 phosphate buffer at pH 7 6 in 0 68 per cent saline   Mixtures of 1 8 ml of brain suspension and 0 2 ml of falling tenfold dilutions of virus, after standing for 3 hours at room temperature, were tested for virus by the intracerebral inoculation of mice   Suspensions of infected mouse or sheep brain provided a source of louping-ill virus   The LD_{50} titre was determined and compared with that of a similar control series of mixtures in a 10 per cent suspension of normal mouse brain   The presence of a neutralizing substance was deduced when the titre was significantly lowered by suspension in infected brain   The result was expressed as the neutralization index, which was the antilogarithm of the difference between the titres (expressed as the logarithms of the dilutions giving the 50 per cent mortality end-points) in the control and test mixtures   The infected brain suspensions were tested both unheated and after heating at 56° C for 30 min before adding the dilutions of virus, in order to destroy any living virus they already contained   It was thus possible to demonstrate a neutralizing substance in brains, even though they originally contained living virus

Brain suspensions from 9 of 12 sheep neutralized virus to a significant degree, the titre of virus being reduced tenfold at least (Table I)   Demonstrable virus was present in the 3 brains with no neutralizing activity, but was absent from 5 of those containing the neutralizing substance   One brain suspension had a neutralization index of 100 , this animal, which had not received an injection of vaccine, had died 7 days after inoculation, the stage of paralysis having lasted 1 day   In spite of having the largest amount of neutralizing substance the brain suspension contained free virus   A suspension of normal sheep brain did not neutralize virus

In two experiments no neutralizing substance was found in the pooled brain suspension of two paralysed mice, examined 8 and 9 days after intraperitoneal inoculation with louping-ill virus   Similarly the brain of a paralysed sheep, killed 6 days after infection by intracerebral inoculation, did not neutralize virus   Four suspensions, each made from the brains, spinal cords and spleens of 5 sheep, infected by the intracerebral route and killed 5 days later, had no neutralizing activity

Tests for neutralizing activity were carried out on suspensions of spleen from three sheep, infected by peripheral inoculation, which had neutralizing substance in their brains   Two of the three suspensions, after being heated to 56° C for 30 min , neutralized virus , before heating one of the positive suspensions contained free virus, but virus was not demonstrable in the other

Properties of the neutralizing substance

Brain suspensions were centrifuged at 3000 r p m in an angle centrifuge for 2 hours and the supernatants were tested for neutralizing activity, allowing 3 hours' contact at room temperature with dilutions of virus The supernatants appeared to neutralize virus to the same degree as the original suspension Neutralizing activity was not affected by filtration of a suspension through a gradocol membrane of 800 mμ A P D Although it withstood heating at 56° C for 30 min , most of the neutralizing substance was inactivated at 70° C for 30 min inactivation was complete at 100° C

Serial dilutions of virus were inoculated intracerebrally into mice which 3 hours previously had received intraperitoneal injections of 1 ml of brain suspension No protection was afforded by brain suspensions which exhibited neutralizing activity *in vitro*

Attempts were made to separate virus from neutralizing substances by the centrifugation of a neutral mixture A series of dilutions of virus in suspensions of brain, after 3 hours' contact at room temperature, were centrifuged for 60 min in the angle centrifuge and the supernatants were inoculated intracerebrally into mice The end-point of the titration was the same as that of mixtures which had not been centrifuged

To 13 5 ml of brain suspension, 1 5 ml of an infected mouse brain suspension, containing 600,000 M L D of virus, was added and the mixture was left for 3 hours at room temperature to allow combination of all the neutralizing substance with virus, there being excess virus present The mixture was then heated at 56° C for 30 min to destroy virus, and afterwards tested for neutralizing activity by the method already described As a control some of the original brain suspension without added virus was allowed to stand for 3 hours, similarly heated and then tested for neutralizing activity against dilutions of virus Both the test and control mixtures effected the same degree of neutralization of virus It appeared that heating had destroyed not only free virus but also the virus which had combined with neutralizing substance, thus setting free the neutralizing substance in a fully active form, able to combine again with freshly added virus

With 4 brain suspensions, which did not contain free demonstrable virus, it was possible to compare the neutralizing activity of materials inactivated at 56° C with that of unheated materials , there was no significant difference Since the neutralizing activity was not greater in the heated suspension, there was no evidence that in the fresh brain virus was present combined with neutralizing substance The method, however, for estimating neutralizing activity was not sufficiently sensitive to detect the small amount of neutralizing substance capable of being liberated by heating if only a little combined virus had been present

These findings suggested that the neutralizing substance was antibody Although it was not possible to test the neutralizing substance against a wide range of viruses and thus demonstrate its specificity, it was shown that a brain suspension which neutralized louping-ill virus did not neutralize the virus of lymphocytic chorio-meningitis

From 6 sheep, whose brains were examined, blood samples were taken just before death and the sera were tested for neutralizing antibodies to louping-ill

rius, using the method described by Edward (1948), except that buffered saline was employed as a diluent instead of serum-broth All the sera contained antibody, but the amount was not proportional to the length of time (between 7 and 14 days) the animal had survived infection (Table I) Also there was

TABLE I —*Results of Testing Brains for Neutralizing Activity*

Sheep No	Interval in days between inoculation and death	Interval in days between onset of nervous signs and death	Whether vaccinated	Presence of virus in brain	Neutralization index of heated brain suspension	Neutralization index of serum
S182	7	1	Not vaccinated	+	100	
S154	11	1	Vaccinated	—	30	
S183	6	1	Not vaccinated	+	30	
49	14	4	,,	—	50	1000
21	12	1	Vaccinated	—	50	800
S157	17	11	,,	—	10	
12	7	< 1	,,	+	10	30
37	8	1	,,	±	10	1500
29	10	2	,,	—	10	
S185	8	1	Not vaccinated	+	< 10	
16	7	< 1	Vaccinated	+	< 10	300
40	7	< 1	,,	+	< 10	60

no correspondence between the neutralizing activities of the serum and of the brain suspensions of a particular animal Although in Table I the degrees of neutralization produced by both serum and brain suspensions are expressed as neutralization indexes, it must be pointed out that different techniques were used to estimate neutralization in the two materials

DISCUSSION

Gard (1944) described an inhibitory substance in the brains of mice, which had developed immunity to mouse poliomyelitis subsequent to infection Mice were inoculated with a strain of virus of low virulence, causing myelitis only, 4 weeks later, when survivors were inoculated with the virulent encephalitic Theiler's FA strain, mice which had showed paralysis proved immune After another 4 weeks their brains were shown to contain an inhibitory substance by titrating mixtures of brain suspension with dilutions of FA virus The infectivity of the mixtures was less than that of similar dilutions of virus mixed with saline or normal mouse brain suspension By centrifugation the greater part of the inhibitory activity was found in the deposit of brain tissue If a mixture of virus and an inhibitory brain suspension was centrifuged in the angle centrifuge dissociation of virus and inhibitor took place, and the supernatant had an infectivity equal to that of the control mixture of virus and saline The inhibitor was also effective *in vivo*, 0 5 ml of brain suspension, injected 24 hours before or at the time of inoculation with virus, caused definite inhibition Gard

concluded that the inhibitory factor was a substance residing in the infected and immune cell itself and was not antibody

The neutralizing substance, found in the brains of sheep infected with louping-ill, differed from that described by Gard, it would appear to be identical with neutralizing antibody Although the neutralizing power of a brain suspension seemed to bear little relation to the level of serum antibody, it appeared that the chances of demonstrating virus in the brain after death were reduced the longer the animal survived after inoculation and the longer the period since the onset of encephalitis, both factors which would favour a high level of antibodies Previous attempts at immunization, even though they failed to protect and were made with feebly antigenic material, also made the isolation of virus less likely

It is well known that antibody in the tissues can make the isolation of virus impossible The findings with louping-ill in sheep show that it may not be possible to isolate virus from the brain, even though it is examined within 24 hours after the first signs of encephalitis and only 10 days after injection of virus They therefore emphasize the difficulties which may attend the diagnosis of neurotropic virus infections by isolation of virus from the brain

<div align="center">SUMMARY</div>

Louping-ill virus could not be demonstrated in 11 out of 30 (37 per cent) brains, obtained from sheep which had succumbed to louping-ill after subcutaneous inoculation with virus

A substance, capable of neutralizing virus *in vitro*, was found in the brains of some sheep infected by the subcutaneous route It appeared to be identical with neutralizing antibody, and did not have the properties of a neutralizing substance, which had been described in the brains of mice infected with mouse poliomyelitis

<div align="center">REFERENCES</div>

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SURVEY OF PAPERS

HERBERT has studied the growth requirements of *Pasteurella pestis* in a chemically-defined medium. He suggests that hydrogen peroxide, formed under aerobic conditions, inhibits growth. Growth is made possible by adding reducing substances which neutralize peroxide or inhibit its formation or by adding haemin which is assumed to promote the synthesis of catalase (p 509)

HAYWARD has shown that the activity of the complement-fixing antigen in suspensions from chick embryos inoculated with herpes simplex virus is chiefly due to a " soluble " specific substance separable from the virus by high-speed centrifugation. The complement fixation technique affords a sensitive method for detecting antibody in human sera (p 520)

MAYER GROSS AND WALKER find that during hypoglycaemia induced by insulin coma therapy there is a decrease in plasma amino acid nitrogen as long as the blood glucose is below 80 mg per 100 ml. They describe experiments which indicate that this effect is related to hepatic deamination (p 530)

SEVITT finds that the protection of guinea pigs against the action of histamine by anti-histamine drugs has no detectable effect on experimental burns of various degrees of severity (p 540)

CAMPBELL finds that the localization of intracellular β-glucuronidase in the mouse kidney and liver is coincident with the mitochondria. Enzymic activity is high in malignant tumours and is mainly localized in anaplastic areas where cell division is prominent (p 548)

GRIESBACH AND KENNEDY have thyroidectomized one pair of parabiotically-joined rats. The thyroids of the intact partners have shown no marked stimulation indicating that the amount of thyrotropic hormone passing from the thyroidectomized to the intact partner has been negligible (p 555)

WILSON SMITH AND HUMPHREY find that sodium salicylate reduces the intensity of passive Arthus reactions in both rabbits and guinea-pigs and of Shwartzman reactions in rabbits but has no appreciable effect on acute anaphylactic shock in guinea-pigs, whereas anti-histamine drugs prevent acute anaphylactic shock but have little effect on the skin hypersensitivity reactions (p 560)

FARAGÓ AND PUSZTAI have followed the responses in rabbits to inoculation of combined diphtheria tetanus-pertussis prophylactic and the same antigens used separately. The most effective preparation was the combined mixture with aluminium phosphate carrier followed by the combined mixture without the mineral carrier. The findings are discussed in relationship to human immunization (p 572)

EDWARD has found a virus-neutralizing substance in the brains of sheep which have succumbed to encephalitis produced by the subcutaneous inoculation of louping-ill virus. The properties of this substance suggest that it is an antibody (p 582)

The anaerobic glycolysis of slices of the parent tumour T 86157, maintained in the solid form, was also not affected by DNP, but a positive effect was observed with cells of the same tumour grown in the ascites form A suspension of free cells prepared from the solid tumour T 86157 showed a high glycolytic rate and DNP stimulated the anaerobic glycolysis provided that preferably less than 7 mg of dry weight of the cells were incubated with the uncoupling agent in a final volume of 1 6 ml bicarbonate buffer

TABLE III —*Effect of 2 4-Dinitrophenol on the Aerobic and Anaerobic Glycolysis of Normal and Neoplastic Tissues* , 10^{-4} M *DNP*

| | | | Lactate production (micromoles) | | | |
| | | | Anaerobic | | Aerobic | |
Tissue	Dry weight (mg)	Incubation period (minutes)	DNP absent	DNP present	DNP absent	DNP present
Ehrlich ascites carcinoma	6 8	20	5 6	13 0	4 2	12 5
	5 2	40	6 5	15 0	4 2	15 5
T 86157 ascites lymphosarcoma	6 0	35	7 8	18 8	4 6	20 8
	3 1	35	4 2	13 5	3 0	13 3
T 86157 solid lymphosarcoma (sliced)	13 0	60	14 9	15 6	6 7	14 3
	6 5	60	8 3	8 9	3 6	7 9
T 86157 solid lymphosarcoma (cell prepn)	12 9	60	20 9	22 3	11 0	20 2
	7 4	60	9 0	11 1	4 4	11 2
	4 2	60	4 3	7 2	2 9	7 7
	4 1	60	3 6	5 1	—	—
	3 4	60	3 5	6 0	3 0	5 8
Strain 3' non malignant lympho- blasts from T 86157	7 8	60	9 2	9 0	6 2	11 9
	8 1	60	10 0	10 8	6 2	13 0
Strain 6 A malignant lympho blasts from T 86157	5 2	60	8 0	9 4	3 3	9 7
	2 8	100	4 7	4 7	1 2	4 9
Bone marrow cells (rabbit)	8 3	60	5 5	6 5	2 2	7 2
Solid tumours (slices)—						
UV 256 mouse sarcoma	15 3	60	8 9	9 8	4 1	8 3
T 28012 mouse hepatoma	15 1	60	7 1	7 7	2 7	5 7
Primary rat hepatoma (hepato- cellular type)	15 9	60	10 2	9 1	5 1	8 5
BY 252 transplanted rat hepa toma (hepatocellular type)	15 0	60	16 0	14 3	8 5	19 0
	16 9	60	20 2	22 8	9 2	18 9

It is seen in Table II and Table III that 10^{-4} M DNP increased the anaerobic glycolysis from 1 5 to 3-fold in the various expriments with the three ascites tumours A nearly two-fold stimulation was observed in most cases with the S_3A ascites tumour (Table II, Experiments 3–5) The marked stimulation of the anaerobic lactate production of the ascites tumour cells observed in the present experiments by DNP is without precedent* (compare Simon, 1953) In the case

* In a recent discussion at the Ciba Foundation Symposium on the Regulation of Cell Metabolism (Churchill Ltd , London 1959) Greville (p 12 and 255) mentioned another instance in which DNP vastly stimulates anaerobic glycolysis (intact frog muscle)

of yeast, for instance, only a very small effect has been noted (Stickland, 1956) However, in the course of preparing the present paper a publication by Clowes and Keltch (1954) came to our attention in which the anaerobic lactate production of sarcoma 180 ascites cells was reported to be stimulated 1 7-fold by dinitrocresol

In an attempt to account for our findings the following hypothesis was proposed (Emmelot and Bos, 1958a) The glycolytic potential of the ascites cells might be so high that even under anaerobic conditions when most of the P_i and ADP of the cells is available to the cytoplasmic glycolysis, the actual rate of the latter process remains below that attainable if the steady-state concentrations of P_i and ADP were to be raised In other words, the anaerobic glycolysis was governed by the (slower) rate of ATP-dephosphorylation Now, DNP is known not only to uncouple the oxidative phosphorylations in the mitochondria but also to induce an active ATP-splitting enzyme which converts ATP into ADP and P_i (DNP activation of the latent ATPase) Activation of the ascites tumour mitochondrial ATPases by DNP might lead to a higher steady-state level of ADP and P_i in the cytoplasm and thus favour the anaerobic glycolysis The following experiments have been conducted in order to obtain support for this hypothesis

Effect of DNP, KCN and methylene blue, alone or in combination, on the glycolysis of the S_3A ascites carcinoma

First, it was checked, by inhibition of the cytochrome oxidase in the presence of KCN, whether the conditions were really strictly anaerobic Aerobic glycolysis in the presence of KCN (10^{-3} M) was indeed found to be similar to the anaerobic glycolysis in the presence or absence of KCN (Table IV , Experiments 1c, 2c and 4c) The effect of DNP on the aerobic and anaerobic glycolysis was also apparent in the presence of KCN (Experiments 2d, 4d) According to expectation methylene blue only abolished the Pasteur effect (Experiments 1d, 1e, 3e)

Anaerobic glycolysis in homogenates of the S_3A ascites carcinoma

Homogenates were prepared as mentioned under Materials and Methods Microscopic examination showed that at the most 10 per cent of the cells had remained intact Moreover, intact cells did not metabolize added fructose diphosphate but the homogenates were very active in this respect

In all the following experiments incubation was performed in the medium according to LePage (1948) from which fructose diphosphate was omitted The medium designated with the capital A contains KF (10^{-2} M) and pyruvate to circumvent the enolase block, whereas in medium B fluoride was absent and only a trace amount of pyruvate was added to prime the triose phosphate dehydrogenase reaction by affording a rapid reoxidation of DPNH In both media glycolysis proceeded from glucose A trace amount of ATP was also present to prime the hexokinase reaction , when no ATP was added glycolysis still proceeded, even in medium B, at a high rate The latter result indicated that enough ATP was initially present to satisfy the hexokinase reaction and, thus, that ATP dephosphorylation was not very high The ease of obtaining a vigorous glycolysis with ascites cell homogenates under the above conditions stands in marked contrast to the situation in other homogenates (e g , from liver) in which it is necessary to add ATP, fructose diphosphate and/or hexokinase to obtain similar results

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TABLE IV —*Effect of 2 4-Dinitrophenol, Potassium Cyanide and Methylene Blue Alone or in Combination on the Glycolysis of S_3A Ascites Carcinoma Cells*

DNP 10^{-4} M, KCN 10^{-3} M, Methylene blue 10^{-4} M
Incubation period   Experiments 1, 2, 3 and 4   45, 30, 45 and 30 minutes ,
mg of dry weight cells   6 1, 5 4, 6 1 and 6 4, respectively
Lactate production in μmoles

Experiment No	Addition	Lactate production	
		Anaerobic	Aerobic
1a	None (control)	12 5	9 4
b	DNP	20 2	21 3
c	KCN	12 2	11 9
d	M Bl	—	11 9
e	KCN + M Bl	—	11 7
2a	None (control)	7 3	5 0
b	DNP	18 9	20 0
c	KCN	—	7 2
d	KCN + DNP	—	16 6
3a	None (control)	12 3	7 5
b	DNP	22 3	23 9
c	M Bl	—	12 1
4a	None (control)	10 4	7 1
b	DNP	17 1	16 9
c	KCN	10 8	9 9
d	KCN + DNP	17 0	17 9

In the chain of reactions which starts with one molecule of glucose and ends with two molecules of lactic acid, two molecules of ATP are needed to phosphorylate glucose to fructose-1,6-diphosphate and in subsequent reactions four molecules of ATP are generated   2 ATP from 2 P_i and 2 ADP by the triose phosphate dehydrogenase system, and 2 ATP from 2 ADP and the phosphate contained in 2 molecules of enolphosphopyruvate   For the purpose of the present discussion the glycolytic reactions may be abbreviated to the following scheme, in which A and B denote the reactions occurring in medium A and B

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{(A, B)}} \text{Glucose-6-P} + \text{ATP}$$

$$\text{Fructose-6-P} + \text{ATP} \xrightarrow{\text{(A, B)}} \text{Fructose-di-P} + \text{ADP}$$

$\left.\right\}$ 2 ATP → 2 ADP

$$\text{3-P-glyceraldehyde} + P_i + \text{ADP} (+ \text{DPN}) \xrightarrow{\text{(A, B)}} \text{3-P-glycerate} + \text{ATP} (+ \text{DPNH}_2)$$

$\left.\right\}$ 2 P_i + 2 ADP → 2 ATP

blocked by KF

$$\text{2-P-glycerate} \xrightarrow{\hspace{2cm}} \text{enolphosphopyruvate}$$
(B)

$$\text{enolphosphopyruvate} + \text{ADP} \xrightarrow{\text{(B)}} \text{enolpyruvate} + \text{ATP} \quad\vert\quad 2 \text{ ADP} \to 2 \text{ ATP}$$

$$\text{(enol)pyruvate} (+ \text{DPNH}_2) \xrightarrow{\text{(A, B)}} \text{lactate} (+ \text{DPN})$$
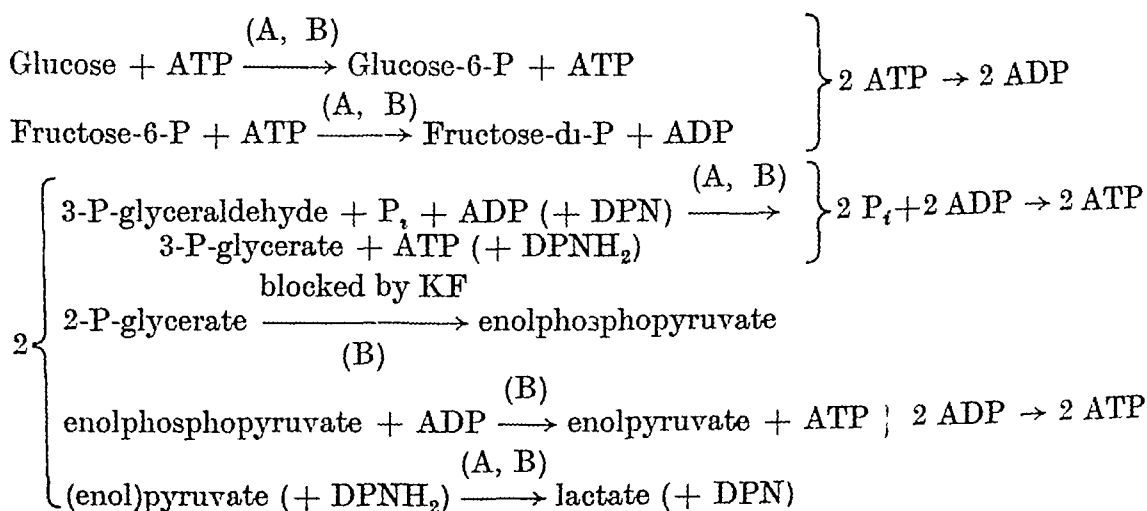
Glycolysis must come to a stop as soon as the available P_i and ADP is converted to ATP   It should, however, be noted that the reaction rate of the triose phosphate

dehydrogenase system decreases already before the P_i and ADP have disappeared completely   The ATPases which split ATP into ADP and P_i and other ATP-consuming processes, are thus important in conditioning the rate of glycolysis   If these activities are very low, glycolysis will proceed sluggishly and soon stop   On the other hand, if ATP dephosphorylation is very high the kinases become deprived of ATP and glycolysis will stop also   Such a situation was encountered by Meyerhof and Wilson (1949) in homogenates of certain solid tumours   Since the kinases and the ATPases compete for ATP, the relative activity of these enzymes will govern the glycolytic rate   It appeared that the hexokinase was very
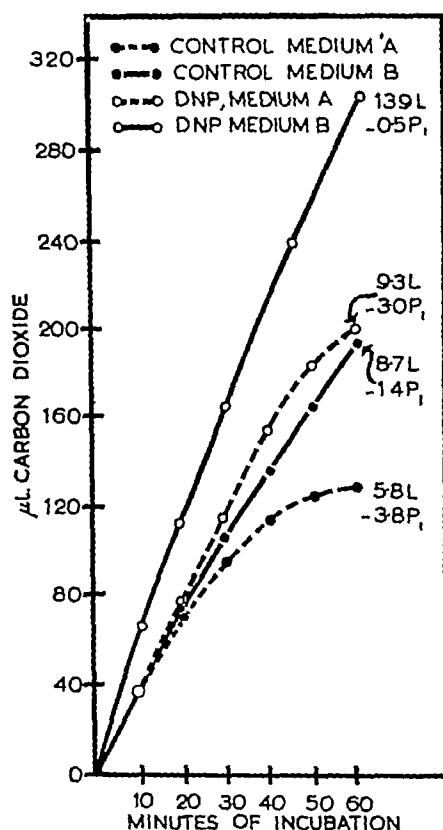

FIG 1 —The effect of 2 4 dinitrophenol on the anaerobic glycolysis of a S_3A ascites carcinoma cell homogenate

Homogenate corresponding with 6 7 mg of dry weight cells 10^{-4} M DNP added as indicated   Medium A and B compare text   L and P_i denote μmoles lactate produced and μmoles inorganic phosphate taken up (−), respectively   3 9 μmoles P_i were present at the start

active in homogenates of the S_3A ascites carcinoma and that ATP dephosphory-lation was not very high   It follows then that in medium A, which contains the ATPase inhibitor fluoride, anaerobic glycolysis will proceed more slowly than in the absence of the inhibitor (medium B)   This was always found to be the case   From Fig 1, which represents one of these experiments, it can be seen that the anaerobic glycolysis in medium A began to slow down after 20–30 minutes and came to a stop after 50 minutes, whereas in medium B glycolysis still continued unhampered   Measurement of the inorganic phosphate showed that in medium A the concentration of P_i had dropped to a low value (0 2 μmole P_i left from the 3 9 initially present) whereas in medium B, in spite of the higher lactate production,

much more P_i (2 5 μmoles) was left. These results strongly suggest that the rate of glycolysis was governed by the available P_i (and ADP), and that, in the presence of fluoride, P_i soon reached a limiting concentration by being continuously converted to 2-phosphoglycerate

TABLE V —*The Effect of P_i, ADP and DNP on the Anaerobic Glycolysis of Homogenates of the S_3A Ascites Tumour*

All quantities in micromoles

Experiment No	Medium (dry weight of cells, time of incubation)	P_i/ADP	DNP (10^{-4} M)	Lactate production	Extra lactate following addition of P_i	ADP	DNP	P_i uptake
1a	A (8 mg, 60')	3 9/—	—	10 4				3 8
b		3 9/—		15 5	6 5			2 6
c		10 9/—	+	16 9		5 1		6 2
d		10 9/—		16 1				5 3
e		10 9/9 6	+	16 3	−0 6	0 8		7 6
f		10 9/9 6		16 6		0 3		6 2
2a	A (6 8 mg, 80')	3 9/—	—	11 4				—
b		3 9/—		16 1	5 1	4 7		—
c		10 9/—	+	16 5		1 1		—
d		10 9/—		17 6	1 2	1 1		—
e		10 9/9 6	+	17 7		1 4		—
f		10 9/9 6		19 1				—
3a	B (9 0 mg, 70')	3 9/—	—	17 6				1 9
b		3 9/—		26 2	4 6	8 6		1 2
c		10 9/—	+	22 2		4 2		3 9
d		10 9/—		26 4	6 6	1 3		3 3
e		10 9/9 6	+	28 8				5 8
f		10 9/9 6		30 1				5 2
4a	B (8 7 mg, 60')	3 9/—	—	16 1	3 9			1 9
b		10 9/—		20 0		5 8		3 6
c		10 9/9 6		25 8				6 2
5a	B (5 2 mg, 40')	3 9/—	—	4 3				1 4
b		3 9/—		8 7		4 4	1 4	0 4
c		7 6/9 6	+	8 1		1 3	0 4	2 4
d		7 6/9 6	+	9 4			2 4	0 8
6a	A (9 mg, 60')	3 9/—	—	7 8	7 5			3 1
b		10 3/—		15 3		5 3	1 6	6 6
c		3 9/9 6	—	9 4			−0 6	2 9
d		10 3/9 6	—	14 7				5 7
e	B (ditto)	3 9/—	—	14 4		4 5	4 5	1 1
f		10 3/—	—	18 9	4 5	3 4	3 4	2 3
g		3 9/9 6	—	18 9				1 1
h		10 3/9 6	—	22 3	3 4			2 1

If this interpretation is correct it should be possible, first, to enhance the anaerobic glycolysis in the presence of fluoride by adding extra P_i to medium A. This was indeed found to be possible (Table V, Experiments 1a–c, 2a–c, 6a–b; compare also Emmelot and Bos, 1958a). Secondly, addition of ADP might have little extra effect on lactate production, since the necessary ADP for the triose phosphate dehydrogenase system is provided by the active kinases, whereas ADP is no longer required for the phosphorylating reaction in which enolphospho-

pyruvate participates because fluoride blocks the enolase reaction and no enolphosphopyruvate can be formed ADP was indeed found to have little effect on the rate of the anaerobic glycolysis in medium A regardless of the P_i concentration (Table V , Experiments 1c–e, 2c–e, 6b–d, 6a–c)

It appeared that the rate of the anaerobic glycolysis could also be enhanced in medium B by addition of P_i (Table V , Experiments 3a–c, 4a–b, 6e–f), though, as might be expected, to a smaller extent than in medium A The glycolysis might be more dependent upon ADP in the absence than in the presence of fluoride, since in the former case the conversion of enolphosphopyruvate to enolpyruvate requires ADP The stimulatory effect of ADP in medium B was consistently observed, not only at high but also at low P_i concentrations (Table V , Experiments 3c–e, 4b–c, 6f–h and 6e–g, respectively)

Effect of 2 4-dinitrophenol on the anaerobic glycolysis of S_3A ascites cell homogenates

Fig 1 and Table V show that the anaerobic glycolysis could also be stimulated by adding DNP (10^{-4} M), instead of P_i and ADP, to the homogenates incubated in medium A or B (Table V, in the presence of KF Experiments 1a–b, 2a–b , in the absence of KF Experiments 3a–b, 5a–b) The enhanced lactate production in the presence of DNP was always accompanied by a diminished uptake of P_i It may be concluded that, as a result of the DNP-activation of the ATPases, the higher P_i or P_i plus ADP concentration (medium A and B, respectively) allowed a more active glycolysis If so, DNP should be without effect when extra P_i (and ADP) is added to the media This was actually found to be so as shown in column 8 of Table V (Experiments 1, 2, 3 and 5) , that the anaerobic glycolysis in medium A was only dependent upon the P_i concentration but in medium B also upon the ADP concentration, is shown by the fact that in the former medium DNP had no effect when the P_i concentration was raised (Experiments 1c–d, 2c–d) whereas under similar conditions DNP still activated the glycolysis in medium B (Experiments 3c–d and similar results), but only to a small extent when extra ADP was added (Experiments 3e–f, 5c–d)

TABLE VI —*Effect of DNP on ATP-dephosphorylation by Intact S_3A Ascites Carcinoma Cells*
Conditions Emmelot et al (1959)

Dry weight of cells (mg)	Temperature of incubation	DNP (10^{-4} M)	μg P released after (minutes)		
			5	10	20
3 6	27°	−	4	10	27
		+	9	15	28
5 8	27°	−	15	20	30
		+	21	30	40
3 8	37°	−	14	25	45
		+	21	35	58

Effect of 2 4-dinitrophenol on ATP dephosphorylation by homogenates and mitochondria of the S_3A ascites carcinoma

It has been shown previously (Emmelot et al , 1959) by measurement of the P_i release from added ATP that DNP actually increases the ATP-dephosphorylation by the ascites cell homogenates and mitochondria isolated therefrom, both at

27° and 37° C In the case of the homogenates, the enhancement of the phosphate release was not due to an effect mediated by the small amount of intact cells which were still present Intact cells incubated in the presence or absence of DNP at 27° and 37° C did not release any phosphate, but did so in the presence of ATP The latter dephosphorylation was but slightly enhanced by DNP (Table VI) It has been reported (Acs, Ostrowski and Straub, 1954) that ascites cells contain an ATPase located at the outer membrane If one assumes that ATP is unable to penetrate into the intact cells, the present results suggest that the extracellular-bound ATPase is but little activated by DNP

TABLE VII —*Effect of Stilboestrol and Thyroxine on the ATP-dephosphorylation by Mouse Liver Mitochondria and S_3A Ascites Tumour Homogenate at 37° C*

Stilboestrol 5 × 10^{-5} M , thyroxine 7 5 × 10^{-5} M Mitochondria corresponding with 25 mg wet weight of mouse liver, ascites homogenate corresponding with 3 mg dry weight of cells

		μg P released after (minutes)		
Enzyme source	Addition	5	10	20
Liver mitochondria	—	22	30	35
	Stilboestrol	41	62	105
	Thyroxine	36	50	93
Ascites tumour homogenate	—	25	42	60
	Stilboestrol	35	82	132
	Thyroxine	32	73	150

TABLE VIII —*Effect of Stilboestrol on the Glycolysis of Intact and Homogenized Ascites Tumour Cells*

Incubation during 45–60 minutes at 37° C

		Lactate production (micromoles)	
Preparation	Addition	Anaerobic	Aerobic
Intact cells (8 2 mg)	—	16 8	10 9
	Stilboestrol	17 8	16 7
Intact cells (6 1 mg)	—	10 5	
	Stilboestrol	16 5	
Homogenate (9 0 mg , medium B)	—	13 9	
	Stilboestrol	19 4	

Effect of Stilboestrol and Thyroxine on Glycolysis and ATP dephosphorylation of intact S_3A ascites cells and homogenates

Stilboestrol and thyroxine had no effect on the latent ATPase activity of mouse liver mitochondria when incubation was carried out at 27° C However, at 37° C a marked stimulation of the ATPases of mitochondria and ascites cell homogenates was observed (Table VII) (The activation of rat liver mitochondrial ATPase by thyroxine at 37° C has also been observed by Maley and Johnson (1957)) The Pasteur effect was completely abolished by stilboestrol (5 × 10^{-5} M) in all experiments (Table VIII) The anaerobic glycolysis of the ascites cells was enhanced (1 5–1 8-fold) by stilboestrol only in a number of experiments with the smaller amounts of cells In homogenates a rise of 1 4–1 5-fold in the anaerobic

glycolysis was noted in the presence of stilboestrol The oestrogen inhibited the oxidation of the ascites cells by approximately 30 per cent in the present experiments (Shacter, 1956 , Emmelot and Bos, 1958b)

The observations of Barker and Lewis (1956) that thyroxine may enhance the aerobic and anaerobic glycolysis of the Ehrlich ascites carcinoma has been confirmed in the present experiments with the S_3A ascites carcinoma The results were, however, irregular, probably as a result of the decreased solubility of the hormone after slight pH changes of the medium

Effect of 2 4-dinitrophenol on the incorporation of [1–^{14}C]leucine into S_3A ascites carcinoma protein under aerobic and anaerobic conditions

If DNP activates the ATPases of the mitochondria of intact ascites tumour cells, it may be expected that the steady-state concentration of glycolytic ATP is lower in the presence of DNP than in its absence This lowered ATP concentration did not inhibit the hexo- and fructokinase reactions, but might have an effect upon ATP-dependent synthetic processes such as amino acid incorporation into proteins The following experiments (Table IX) show this to be the case The incorporation of [1–^{14}C]leucine into the proteins of the ascites cells was of the same order in the presence or absence of glucose under *aerobic* conditions Mitochondrial ATP, generated by the endogenous respiration, could thus completely satisfy the energy requirements of the amino acid incorporation processes (compare Emmelot and van Vals, 1957) Addition of DNP inhibited leucine incorporation, while addition of glucose counteracted the latter inhibition but could not restore the rate of incorporation reached in the absence of DNP Incorporation of leucine under *anaerobic* conditions was dependent upon the presence of glucose (glycolytic ATP) With glucose present, addition of 10^{-4} M DNP consistently resulted in an inhibition of about 50 per cent of the anaerobic amino acid incorporation Similar results were obtained with [1–^{14}C]leucine added in the range of 0 3–1 0 μmole

It is of interest to compare the present results with those obtained with the Ehrlich ascites carcinoma Since the anaerobic glycolysis of the Ehrlich ascites carcinoma was stimulated by DNP in our experiments (Table III), the amino acid incorporation into the protein of these cells should be depressed under the latter conditions It has actually been reported that DNP, under anaerobic conditions and in the presence of glucose, inhibited the amino acid incorporation into the latter cells by 30 per cent (Rabinowitz, Olson and Greenberg, 1955) However, Bickis, Creaser, Quastel and Scholefield (1957) observed only a slight inhibitory effect The latter result might have been due to the " quality " of their cells , cells which are not in an optimal condition (e g as a result of repeated washings) might already show activated ATPases

Our hypothesis demands that in those cases in which DNP does not enhance the anaerobic glycolysis, the amino acid incorporation under anaerobic conditions in the presence of glucose should not be affected either This was found to be so in experiments carried out with slices of the mouse sarcoma UV 256 (Table IX , Experiment 6)

COMMENT

The present results furnish strong evidence for the supposition that the anaerobic glycolysis of ascites tumour cells may be raised by activation of the intracel-

lular, probably mitochondrial, ATPases The ascites cells apparently possess an enormous capacity to glycolyse, which under normal conditions cannot be attained anaerobically as a result of the relatively slow ATP-dephosphorylation From the experiments on leucine incorporation and those conducted with the homogenates it is concluded that under anaerobic conditions the ATP-turnover increased and its steady-state concentration decreased when DNP was present

TABLE IX —*Effect of 2 4-Dinitrophenol on the Incorporation of [1–^{14}C] Leucine into the Proteins of the S_3A Ascites Carcinoma under Aerobic and Anaerobic Conditions in the Presence and Absence of Glucose*

Incubation in Krebs-Ringer phosphate(*) or bicarbonate(**) buffer aerobically and anaerobically (gas phase 100% O_2 and 100% N_2, 95% O_2 + 5% CO_2 and 95% N_2 + 5% CO_2, respectively), DL-[1–^{14}C]-leucine (0 28 μmoles containing 0 33 μc)

Experiments 1, 2 300 mg wet weight of cells in 5 ml 0 02 M phosphate buffer, 6 mg glucose, 60 minutes

Experiments 3, 4 200 mg wet weight of cells in 5 ml 0 02 M phosphate (or HCO_3) buffer, 3 mg glucose, 30 minutes

Experiment 5 200 mg wet weight of cells in 6 ml 0 03 M phosphate (or HCO_3) buffer, 6 mg glucose, 30 minutes

Experiment 6 300 mg wet weight of slices of sarcoma UV 256 in 3 ml buffer, 3 mg glucose, 30 minutes

Experiment No	Glucose	DNP	Protein specific activity (c /m)			
			Anaerobic incubation		Aerobic incubation	
			(*)	(**)	(*)	(**)
1, 2	—		84		870	
	—	5 10⁻⁵	31		445	
	—	10⁻⁴	14		206	
	+		907		945	
	+	5 10⁻⁵	497		537	
	+	10⁻⁴	339		421	
3, 4	—		40	74		
	—	10⁻⁴	10	15		
	+		588	812		
	+	10⁻⁴	266	427		
5	—		45	81	629	715
	—	10⁻⁴	20		106	124
	+		639	859	576	708
	+	10⁻⁴	326	428	315	371
6§	—			35		
	—	10⁻⁴		35		
	+			314		
	+	10⁻⁴		322		

§ Experiment carried out with slices of the mouse sarcoma UV 256

In bone marrow cells, non-malignant and malignant lymphoblasts and slices of solid tumours no effect of DNP on the anaerobic glycolysis could be demonstrated A variety of reasons may be responsible for this lack of effect The glycolytic capacity may be smaller and the relative rate of ATP-dephosphorylation may be higher in the latter cells and tissues than in the ascites cells Furthermore

the rate of penetration of glucose may limit the glycolysis In the case of tissue slices mechanical factors may be involved Since the entrance of glucose may occur by an active transport mechanism, the number and the specific activity of the active sites in the cell membrane may also be of importance

It has been shown that glucose penetrates into ascites tumour cells very rapidly and that the rate of glycolysis is not governed by the rate of glucose entrance (Crane, Field and Cori, 1957 , Cori, 1956) The generally high glycolysis of ascites as well as of solid tumour cells may be due to this phenomenon, the relative differences between the two types being dependent on the unicellular or multicellular organisation (Hechter, 1957, Bloch-Frankenthal and Weinhouse, 1957)

It has been suggested (Lynen, 1958) that glucose uptake is dependent upon glycolytic ATP The decreased aerobic glucose phosphorylation has been explained (Chance and Hess, 1956 , Lynen, 1958) by assuming that the ATP which is generated under aerobic conditions in the mitochondria is retained and does not readily equilibrate with the cytoplasm As a result the hexo- and fructokinase of the glycolytic pathway should be inhibited as compared with the situation under anaerobic conditions or under aerobic conditions with DNP present, in which more ATP is formed in the cytoplasm However, the finding that the anaerobic glycolysis of the ascites cells can be markedly stimulated by increasing the dephosphorylation of glycolytic ATP, indicates that neither the uptake of glucose nor its phosphorylation is impaired by decreasing the steady-state concentration of glycolytic ATP of ascites tumour cells under anaerobic conditions

SUMMARY

1 The effect of DNP on the aerobic and anaerobic glycolysis of ascites tumour cells, tumour slices and several types of normal and malignant cell suspensions has been studied

DNP abolished the Pasteur effect in all cases The anaerobic glycolysis of the Ehrlich and S_3A ascites carcinomas and the T 86157 ascites lymphosarcoma was found to be stimulated by DNP (10^{-4} M) about 2-fold in general, but higher increases were also observed occasionally No effect of DNP on the anaerobic glycolysis of tumour slices (including the solid T 86157 lymphosarcoma), (non-) malignant lymphoblasts (derived from T 86157 by subculture in vitro) and bone marrow cells was observed The anaerobic glycolysis of cell suspensions prepared from the solid T 86157 lymphosarcoma was enhanced by DNP under certain conditions

2 The anaerobic glycolysis of homogenates prepared from the S_3A ascites carcinoma was markedly dependent upon the concentration of P_i or P_i plus ADP, depending on the experimental conditions A significant rise in the anaerobic lactate production and a drop in the P_i uptake by the homogenates in the presence of DNP was observed DNP exerted no effect when the concentration of P_i (and ADP) in the medium was raised

3 DNP stimulated ATP-dephosphorylation by homogenates and isolated mitochondria of the ascites tumour cells, but not of intact ascites tumour cells, both at 27° and 37° C

4 Stilboestrol had a similar, though smaller, effect as DNP

5 The energy (ATP) derived from the endogenous (fatty acid) oxidation of the ascites tumour cells was equivalent to that derived from aerobic glycolysis plus glucose oxidation, or from the anaerobic glycolysis, in sustaining amino

acid incorporation into the proteins of the ascites cells   DNP inhibited amino acid incorporation into the protein of the S_3A ascites carcinoma under anaerobic conditions in the presence of glucose

6 It is concluded that under anaerobic conditions, when nearly all the P_i and ADP is available to the cytoplasmic glycolysis, the rate of glycolytic-ATP generation by the ascites tumour cells is higher than the rate of ATP-dephosphorylation , as a result the anaerobic glycolysis operates below its potential capacity In the presence of DNP the mitochondrial ATPases are activated and the higher steady-state concentrations of P_i and ADP, resulting from the splitting of glycolytic ATP by the activated mitochondrial ATPases, allow glycolysis to proceed at a higher rate than in the absence of DNP   However, since the increased ATP turnover in the presence of DNP induces a fall in the steady-state concentration of ATP, ATP-dependent anabolic processes of the ascites tumour cells, such as amino acid incorporation, are inhibited under anaerobic conditions in the presence of glucose and DNP

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THE CONTENT OF DIPEPTIDASES AND ACID PROTEINASES IN THE ASCITIC FLUID OF MICE WITH ASCITES TUMOURS

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ATTEMPTS are being made to investigate whether proteolytic enzymes, possibly liberated from the tumour cells, are implicated in the observed extracellular proteolysis of fibrous host proteins at the periphery of malignant tumours (Sylvén, 1945 and 1954, Sylvén and Malmgren, 1957) The solid mouse tumour materials did not, until very recently, allow separate sampling of the extracellular fluid for enzymic assays Therefore, the ascites tumour material was chosen since in this case an extracellular fluid compartment could easily be sampled The present line of approach is supported by some recent evidence that malignant cells in general are more permeable than normal non-malignant cells in the sense that cytoplasmic proteins and enzymes are more freely passing out of the cell membranes The particular physiological conditions and states of adaptation favouring such a liberation are not yet understood

This report summarizes serial data on the total " over-all " dipeptidase and acid proteinase activities of (*a*) the ascites tumour cells, (*b*) the cell-free ascites fluid, and (*c*) the corresponding blood plasma activity levels in the same tumour-bearing mice The particular acid proteinases of normal blood plasma active in the pH range 2 to 6 have been tentatively characterized as belonging to the pepsin and cathepsin groups (Mirsky *et al*, 1952) The changes in plasma dipeptidase and acid proteinase activities in the course of ascites tumours have been described (Ottoson and Sylven, 1959) The present results are, however, burdened by several limitations restricting our conclusions In view of the transport problems involved, the expected more or less non-specific host reactions, and the dilution effects in the different compartments, more direct evidence is needed as to the basic permeability problems of the tumour cells referred to above

PREVIOUS DATA ON THE PERTINENT ENZYMIC ACTIVITIES

Blood serum and plasma —It is well known that normal plasma contains a considerable pool of various peptidases active against commonly tested di- and tripeptides (Grassmann and Heyde, 1929, Fruton, 1946, Fleisher and Butt, 1953)

The dipeptidase activity of mouse plasma against alanylglycine (AG) undergoes a continuous increase in the course of ascites tumour growth (Ottoson and Sylvén, 1959) An endopeptidase activity in rabbit serum against benzoylglycineamide at pH 5 4 was further described by Fruton (1946) In addition, normal mouse plasma contains at least two types of proteinases with activity optima against

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urea-denatured haemaglobin at pH 3 5 and 4 5 (Fig 3) These proteinase activities undergo characteristic changes in the course of ascites tumour growth (Ottoson and Sylvén, 1959)

The total dipeptidase activity referred to above is of a moderate magnitude in mouse plasma (about 0 2 μl 0 1 M NaOH per μl plasma per hour), while the proteinase activities at pH 3 5 and 4 5 are high (extinctions about 0 15 per μl per 90 minutes at 37°) as compared with those of human plasma The cellular sources of these enzymes are not known in detail It seems likely that the proteinase activity at pH 3 5 is due to pepsinogen (activated) from the stomach mucosa (Mirsky *et al*, 1952)

Lymph —Lymph enzymes in general present lower concentrations per volume than blood plasma (Yoffey and Courtice, 1956) The concentration of enzymes is said to run largely parallel with the concentration of proteins in the larger lymph trunks so far investigated A trypsin-like proteinase in thoracic duct lymph of the dog was described by Osato (1921), and a dipeptidase in rabbit leg lymph by Ishino (1935/38) The enzymic content of the smaller efferent lymph channels is not known, however, according to Doyle (1955), lymph from the appendix has a slightly higher peptidase level than blood plasma No data on the content of acid proteinases are available

Interstitial tissue fluid —Little is known of the enzymal activities in the interstitial fluid of normal tissues The concentrations are expected to be of a similar order of magnitude as that in lymph An aminopeptidase activity has been observed by histochemical methods in the oedema fluid of skin disorders in man (Braun-Falco, 1957), and also at the periphery of several malignant tumours (Burstone, 1956) A proteinase activity was long ago postulated to occur in the extracellular fluid of healing wounds explaining the removal of pre-formed fibrous proteins It is not known, however, whether these activities are due to cell damages or if they occur also under normal *in vivo* conditions It was cursorily mentioned by Libenson and Jena (1957) that the cell-free tissue fluid within certain transplanted human tumours contained a greater proteolytic activity at pH 4 than the normal subcutaneous tissue fluid distant from the tumour site Quantitative assays of the peptidase and catheptic activities of the extra-cellular fluid taken *in vivo* from various solid mouse tumour transplants (Sylvén, unpublished) distinctly indicate that these activities per unit volume are con-siderably higher than the levels observed in normal lymph and plasma

Preliminary data on the normal peritoneal fluid collected by capillary tech-niques from the intact peritoneal cavity of the same strain of inbred mice, as used in this report, are being collected Samples pooled from a large number of mice showed a rather high total dipeptidase figure (about 4 times the correspond-ing plasma activity) The pH 3 5 and 4 5 proteinase activities against urea-denatured haemoglobin were of the same order as those of the corresponding blood plasma The relative activities are for comparison included in Fig 7

Ascites tumour fluid —The dipeptidase activity in the fluid of mouse ascites tumours was reported to be low (Malmgren, Sylvén and Révész, 1955) The presence of a carboxypeptidase inhibitor in the fluid was demonstrated by Fein-stein and Ballin (1953), and later on also noted against the AG peptidase by Malm-gren, Sylven and Revész (1955) With reference to the proteinase activities in ascites tumour fluid little is known Spontaneous hydrolysis (pH unknown) of the inherent proteins did not occur during two hours' incubation time according

to Christensen and Riggs (1952), this possibly excludes the presence of marked tryptic activities Malmgren, Sylvén and Révész (1955) cursorily mentioned that a low proteinase activity was noted against nitrocasein and edestin at pH 5 3 and 3 8, respectively The method used at that time was rather insensitive and therefore no significant changes in the proteinase activity were noted in the course of the ascites tumour growth More data are available on the presence of other hydrolytic and glycolytic enzymes in the ascites fluid (Warburg and Christian 1943, Kun, Talalay and Williams-Ashman, 1951, Schade, 1953, Wu and Racker, 1958, Bosch, 1958)

Ascites tumour cells—The peptidase activities against four tested dipeptides and one tripeptide have been described in homogenates from three different ascites tumour cell strains (Malmgren, Sylvén and Révész, 1955) Serial data on the tumour cells at different times after inoculation indicated that the dipeptidase activity was proportional to the protein content of the cells During the same time the average catheptic activity per tumour cell at pH 3 8 against acid-denatured haemoglobin remained low and fairly constant A more detailed study of the cellular proteinases of tumours was recently published (Sylvén and Malmgren, 1957) The present report supplements previous ascites tumour data mentioned above

MATERIALS AND METHODS

Mice—Male backcross mice were used, produced by mating males of the DBA strain with (C3H × DBA)F$_1$ hybrid females The animals were 3 to 4 months old and weighed 16–22 g They were given a standard compressed diet and drinking water *ad libitum*

Tumour strain—The hyperdiploid Ehrlich ascites tumour was employed, referred to as ELD (Bayreuther, 1952, Hauschka *et al*, 1957) The tumour has been maintained by weekly serial ascitic transfers in DBA × (DBA × C3H) backcross mice The transfers were carried out routinely by injecting 0 1 ml of the undiluted ascitic fluid intraperitoneally

Measurement of ascites tumour growth—The total volume of peritoneal fluid and the total number of tumour cells was measured by determining the dilution of an intraperitoneally injected dye (bromosulphthalein), together with total and differential cell counting and determination of the packed cell volume The details of this procedure have been described by Révész and Klein (1954)

Sampling—Each experiment was performed with ascites fluid and cells pooled from three identically treated mice in such a way that each mouse contributed an equal volume of ascites fluid and an equal number of cells Pooled material was used in order to avoid most of the individual variations The ascites fluid and cells was taken out in 3 5 per cent sodium citrate to 1/5 of the total sample volume The fluid and the cells were separated before pooling by centrifugation for 10 minutes at about 600–800 × g The first supernatant was further centrifuged for 10 minutes at about 6000 × g in order to remove all cell debris. Some fluid samples were carefully controlled under the phase-contrast microscope and no cell elements remained After pooling, the supernatants were diluted 2 to 5 times with a 0 05 per cent aqueous solution of sodium deoxycholate according to our standard procedure (Sylvén and Malmgren, 1957)

The cells were washed twice in saline and then resuspended in twice their volume of saline After counting the number of cells in each sample they were

pooled as described above The pooled cell suspension was homogenized for 15 minutes under cooling Sodium deoxycholate was added to obtain a suitable concentration and the mixture was left to extract for one hour at room temperature A cell concentration corresponding to 80 × 10⁶ per ml was used for the proteinase assays and 2 to 4 × 10⁶ per ml for the dipeptidase tests

The average ascites cell volume of the packed cells has been 25 per cent of the total ascites volume This figure has been used for the calculation of the fluid content obtained by centrifugation

Enzymic assays —The dipeptidase assays by titration according to the modification of the Linderstrom-Lang and Holter method have been described by Sylvén and Malmgren (1957) Since the ascites peptidases may be activated by magnesium ions, $MgSO_4$ has been added to a final concentration of 0 006 M

The proteinase determinations using urea-denatured bovine haemaglobin as a substrate have been made according to Ottoson and Sylvén (1959) All samples have been assayed both with and without the addition of cysteine to a final concentration of 0 003 M

Since the enzymic activity data of the fluid compartments are discussed as matters of transport equilibria all results are primarily given per μl fluid

Protein determination —The protein contents have been assayed in triplicate tests with a micro-Kjeldahl method The standard deviation among tests performed on the same material was of the order of 3 per cent and the error of the mean of the three tests was of the order of 2 per cent

RESULTS

The multiplication of the ELD ascites tumour cells was determined in two separate experiments each comprising 30 mice inoculated with 20 × 10⁶ tumour cells At daily intervals, two mice were killed and the geometric mean was calculated for each lot of total cell numbers, cell concentrations per ml ascitic fluid, and amount of cell-free ascitic fluid (four individual determinations from two separate experiments) Some data of this series have been published earlier (Hauschka *et al*, 1957)

Fig 1A shows the increase in total number and concentration of free tumour cells in the ascitic fluid during 12 days of growth after inoculation The growth of the ELD cells is characterized by a continuously increasing generation time The decrease in the growth rate proceeds smoothly and the number of free tumour cells continues to increase until a total number of about 10⁹ cells is reached The concentration of the tumour cells per ml fluid remains largely constant during the major part of the growth cycle and shows only a moderate random variation around a value of about 200 × 10⁶ cells per ml ascites Fig 1B shows the increase in the amount of the cell-free ascitic fluid Since the mean volume of the ELD cells has been found to be fairly constant (about 1360 cubic μ) due to the constancy of the cell concentration, the increase in the amount of the cell-free ascitic fluid is directly proportional to the increase of the total number of tumour cells

The previously reported increase in the average amount of protein per cell, as observed in the ELD and Krebs 2 ascites tumours (Malmgren, Sylvén and Revész, 1955), was corroborated also in this series of the ELD strain. The protein figure rose from an average of 1 4 μg per 10,000 tumour cells, at the fourth day after inoculation, until about 2 8 micrograms at the eighth day, which is at variance with data by Ledoux and Revell (1955)

Protein content of blood plasma and ascites fluid —In our stock of mice the plasma protein concentration went down from a normal level close to 6 per cent to below 4 per cent in the course of the ascites tumour growth (Fig 2) This figure also shows that the protein concentration of the cell-free ascites fluid declines from about 4 5 per cent to about 2 5 per cent at the end of the growth
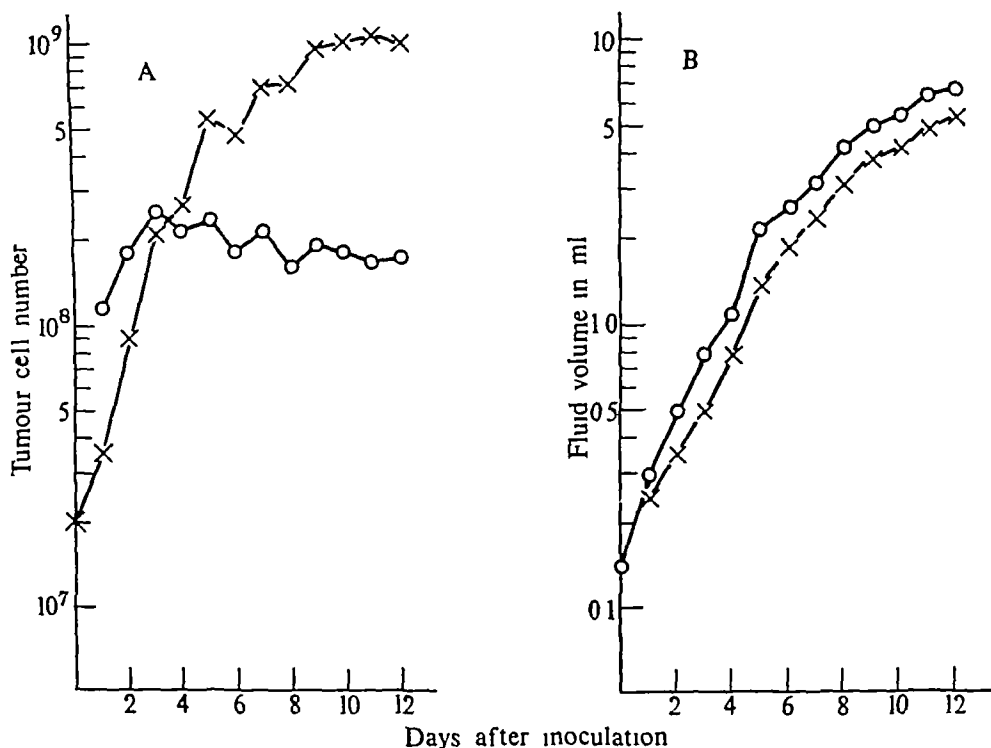

Fig 1 —Changes in the tumour cell numbers and ascites fluid volume plotted against the time after tumour inoculation (cf data by Hauschka *et al*, 1957)

```
1, A —Average total tumour cell numbers      X———X———X
         Tumour cell concentration            O———O———O
1, B —Volume of cell free ascites fluid        X———X———X
         Total volume of ascites               O———O———O
```

curve   Ledoux and Revell (1955) previously found in the same strain of ascites tumours an increasing protein concentration in the fluid up to about 2 per cent at the 14th day of growth   The protein content of normal rabbit lymph (hind leg) is about 2 6 per cent (Benson, Kim and Bollmann, 1955) and of human ascites fluid in cases of liver cirrhosis about 3 per cent (Schoenberger *et al* , 1956)

Albumin-globulin ratio of the ascites fluid —The relative amounts of albumin and globulins assayed by the electrophoretic method* were as follows

	Albumin (%)	Globulins (%)	A/G ratio
Normal male mouse plasma (inbred stock)	43	57	0 75
Plasma of ascites tumour bearing male mice	50	50	1 0
Ascites tumour fluid	56	44	1 25

* The determinations have kindly been made in the laboratory of Dr B Olhagen, at the Rheumatic Clinic Karolinska Hospital Stockholm

The relative excess of the albumin fraction in the ascites fluid agrees with the figure of Kun *et al* (1951), who found about 58 per cent albumin in pooled Ehrlich ascites tumour fluid   The A/G ratio in the ascites fluid is thus 1 25, which is significantly larger than that of normal mouse plasma
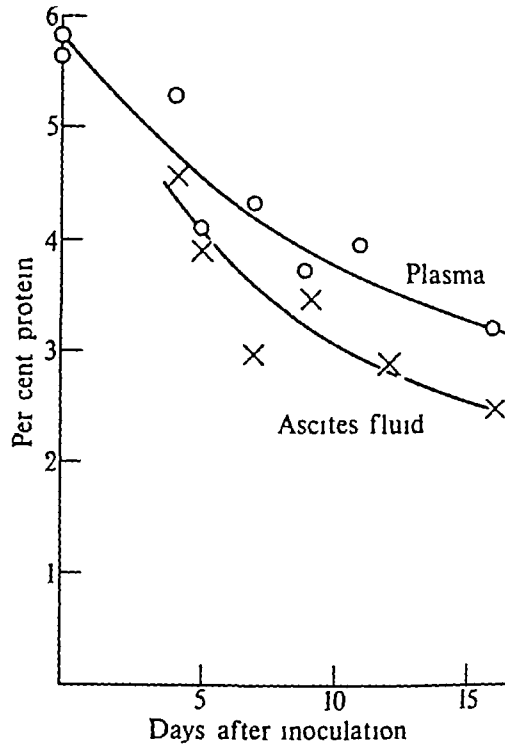

FIG 2 —The change in protein content of mouse blood plasma and cell free ascites tumour fluid with the time after inoculation   Each figure obtained from samples pooled from three mice (see text)
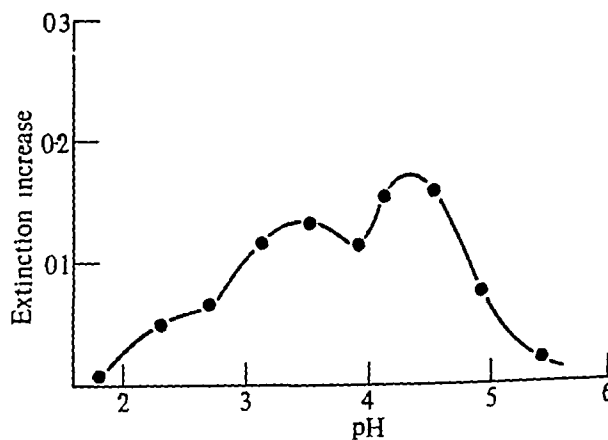

FIG 3 —The pH activity curve of normal mouse plasma using urea denatured haemoglobin as a substrate , cysteine added (see text)   All extinctions are given per μl plasma   The above activity optima will become displaced to the left when acid denatured haemoglobin is used (Ottoson and Sylvén, 1959)

Enzymic activities of the ascites fluid and tumour cells —From about the 12th day of ascites tumour growth the dipeptidase activity of the ascites fluid rapidly

increased (Tables I and III) This activity of ascites fluid did coincide with the late increase in dipeptidase activity of the blood plasma of the same tumour-bearing mice (Ottoson and Sylvén, 1959) Maximum activity figures (in the presence of the dipeptidase inhibitor, previously mentioned) at the end of the growth period were equivalent to about 1 8 μl NaOH, which is more than three times the corresponding plasma level The recorded extracellular activity constitutes from 0 5 to 4 per cent of the total activity contained per μl ascites inclusive of the tumour cells
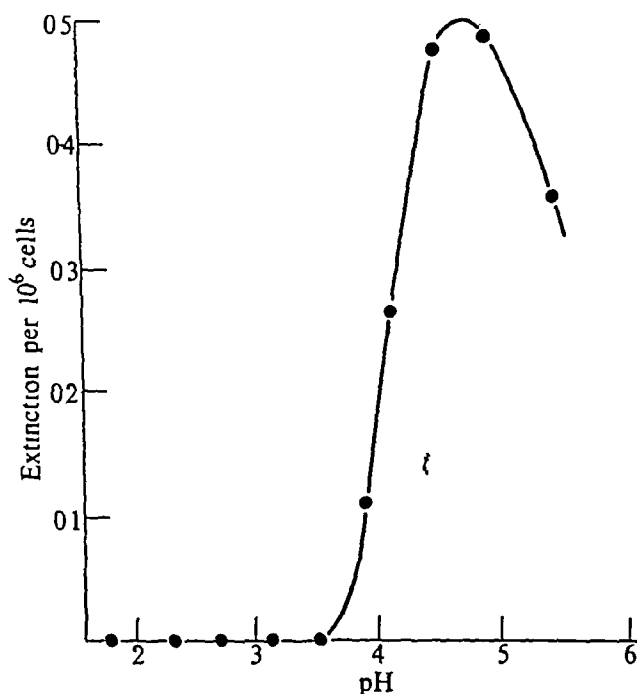

Fig 4 —The pH activity curve of the acid proteinases of washed ascites tumour cells pooled from three mice on the fifth day of tumour growth Substrate urea denatured haemo globin , cysteine added Extinctions expressed per 10⁶ cells

In the case of the proteinases active in the pH range of 2 to 6 the conditions are more complicated In normal mouse plasma there are at least two (possibly three) proteinases, which show optimum activity against urea-denatured haemoglobin at pH 3 5 (3 5 enzyme) and 4 5 (4 5 enzyme) (Ottoson and Sylvén 1959 , Fig 3) The first one is probably a pepsin-like enzyme (Mirsky et al , 1952), while part of the second one is expected to be of cathepsin nature The acid proteinases of lymph have not been studied It may further be added that the extracellular fluid obtained in diluted form from washings of the peritoneal cavity of normal mice also contains two proteinase activity peaks of about equal magnitude at pH 3 5 and 4 5 against urea-denatured haemoglobin (unpublished data) The shape of this pH-activity curve is rather similar to that of blood plasma, but distinctly different from that of the tumour ascites fluid (Fig 5) mentioned below In the interstitial fluid of solid mouse tumour transplants two proteinase peaks have also been observed at about pH 3 8 and another at 4 5–4 7 (Sylvén, unpublished data) The pH-dependence of the ascites tumour cell proteinases is shown in Table I and Fig 4, which demonstrate that the pH 3 5 enzyme is lacking,

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while, instead, enzymes of the cathepsin group with optimum activity around pH 4 5 are present   On the whole, the cathepsin activity is low in the tumour cells (Malmgren, Sylvén and Révész, 1955 , Sylvén and-Malmgren, 1957)   The appearance of some pH 3 5 proteinase in the tumour cell homogenates at the end of the growth period (Table I) is considered to be caused by an admixture of proteins from the ascites fluid not removed by our saline washing
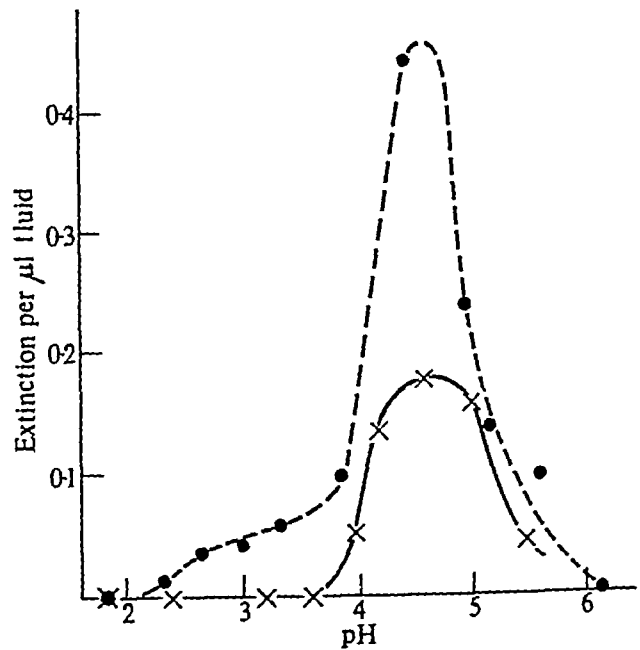

FIG 5 —The pH activity curve of acid proteinases contained in fresh cell free ascites fluid   Conditions the same as in Fig 3   Data from three pooled samples of five days old (×——×——×) and twelve days old (●— — — —●) ascites tumours

TABLE I —*Serial Data on the Total Dipeptidase and Proteinase Activity of Ascites Tumour Cells*

Each figure obtained on materials pooled from 3 inbred male mice

| | | Proteinase activity Extinction increase per 10^6 cells | | | |
| | | Cysteine added | | Cysteine not added | |
Age of tumour (in days)	Dipeptidase activity (μl 0 1 N NaOH per 10^4 cells	pH 3 5 E	pH 4 5 E	pH 3 5 E	pH 4 5 E
4	—	0 15	0 30	0 02	0 32
5	—	0 00	0 94		0 70
7	0 7	0 00	0 84	0 07	2 16
9	2 9	0 00	2 10	0 14	0 64
12	2 5	0 09	0 72	0 15	1 60
16	2 0	0 14	1 46	0 23	

The ascites tumour fluid, on the other hand, shows certain changes in the pH distribution curve as compared with that of normal mouse blood plasma   During the early phase of tumour growth the pH 1 8–3 5 enzymes are depressed in activity or almost lacking (Fig 5 and 6), while a 4 5 enzyme of cathepsin-type (thermo-

and acid-labile) is distinguished   In the course of the later stages of tumour growth the pH distribution gradually changes, more of the pH 3 5 enzyme appears, and further the ratio between the extinction maxima at 3 5 and 4 5 is quite
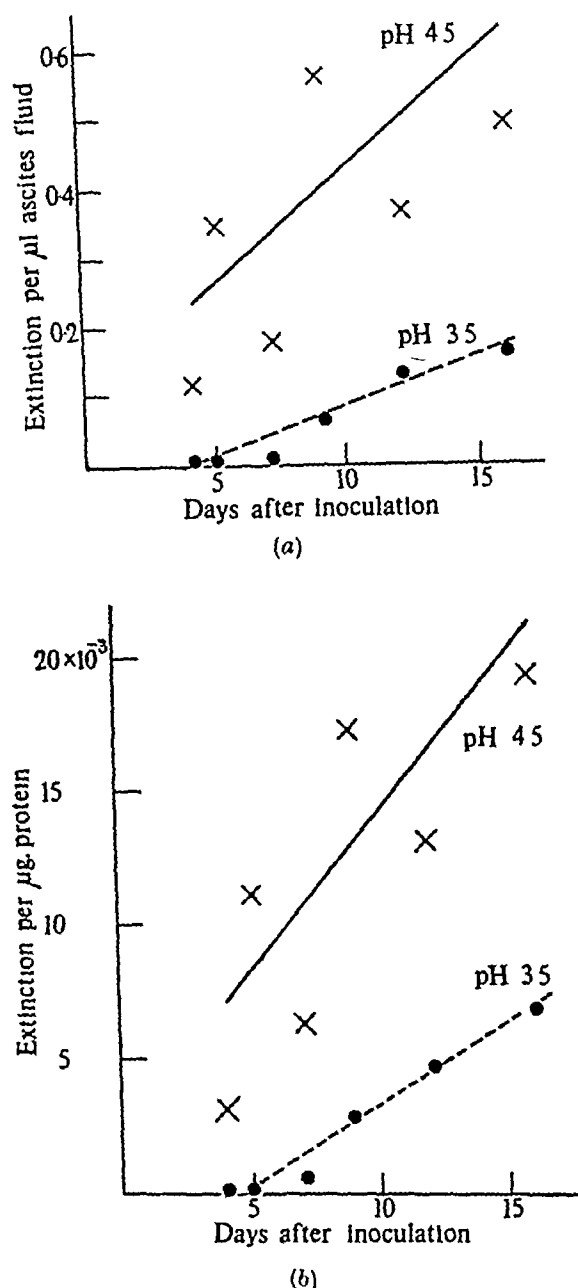

(a)

(b)

Fig 6 —The observed variations in the acid proteinase activity of fresh cell-free ascites fluid at the pH optima 3 5 and 4 5   Each figure obtained from three pooled samples Activities expressed per µl fluid (6, A) as well as per µg protein content (6, B)

different as compared with that in the blood plasma   Fig 6, A and B, demonstrate the continuous increase in both activities in the later course of ascites tumour growth   The pH 3 5 enzyme in the ascites fluid does not reach the corresponding

plasma level of tumour-bearing mice at the end of tumour growth (Ottoson and Sylvén, 1959)

TABLE II—*Serial Data on the Dipeptidase and Proteinase Activity per µl Ascites Fluid Pooled from 3 Inbred Male Mice*

Age of tumour (in days)	Dipeptidase activity (µl 0 1 M NaOH per µl fluid)	Proteinase activity Extinction increase per µl fluid			
		Cysteine added		Cysteine not added	
		pH 3 5 E	pH 4 5 E	pH 3 5 E	pH 4 5 E
4	0 53	0 01	0 12	0 01	0 08
5	—	0 00	0 32	—	—
7	0 43	0 01	0 18	0 03	0 14
9	0 31	0 07	0 58	0 08	0 52
12	0 74	0 14	0 36	0 15	0 30
16	1 8	0 17	0 48	0 22	0 54

TABLE III—*Serial Data on the Dipeptidase and Proteinase Activity per Protein Content in Ascites Fluid Pooled from 3 Inbred Male Mice*

Age of tumour (in days)	Per cent protein content	Dipeptidase activity (µl 0 1 M NaOH per mg protein)	Proteinase activity Extinction increase per mg protein			
			Cysteine added		Cysteine not added	
			pH 3 5 E	pH 4 5 E	pH 3 5 E	pH 4 5 E
4	4 7	5 4	0 2	3 0	0 3	1 8
5	3 9	—	0 0	11 0	—	—
7	2 9	14 7	0 5	6 2	1 1	4 6
9	3 4	9 2	2 3	17 2	2 5	15 8
12	2 8	44 6	4 8	13 0	5 2	10 8
16	2 5	72 0	6 7	19 2	8 5	21 0

So far all activities have been expressed per µl ascites fluid and plasma The observed increases in enzymic activity are still more marked when correlated to the protein content of the compartments (Table III)

DISCUSSION

The observed enzymic data illustrate the net results of a complex series of transport and permeability events *in vivo*, which seem difficult to distinguish in detail Since the enzymes are unstable and are possibly subject to different degrees of dilution and inactivation in all the tissue compartments concerned the conclusions are necessarily vague, and have to be supported by other more direct and independent data The discussion will mainly be based on the enzymic activities per unit volume as is usual in problems involving transport stable markers Additional information may be obtained by comparing the enzymic activities on a per-protein basis

It is further assumed that there is a rapid exchange of water and a slower exchange of protein constituents between the ascites fluid and blood mainly via the lymphatic pathways (Courtice and Steinbeck, 1950 , Prentice, Siri and Joiner, 1952 Abdou, Reinhardt and Tarver, 1952 , McKee *et al*, 1952 , Berson and Yalow, 1954 , and Schoenberger *et al*, 1956) It is further presupposed that the

ascites fluid originates from the blood serum (Straube, 1958), but also that certain ionic and enzymic contributions may appear from the interstitial fluid of the peritoneal walls as well as from the intracellular compartment of the peritoneal cells, normal as well as tumorous ones

With reference to the exchange of proteins between the blood and ascites fluid present data seem to justify the conclusion that there is a " restricted diffusion " of globulins into the peritoneal cavity leading to the observed shift in the A/G ratio This might indicate a certain " sieving-effect " on the part of the capillary membrane
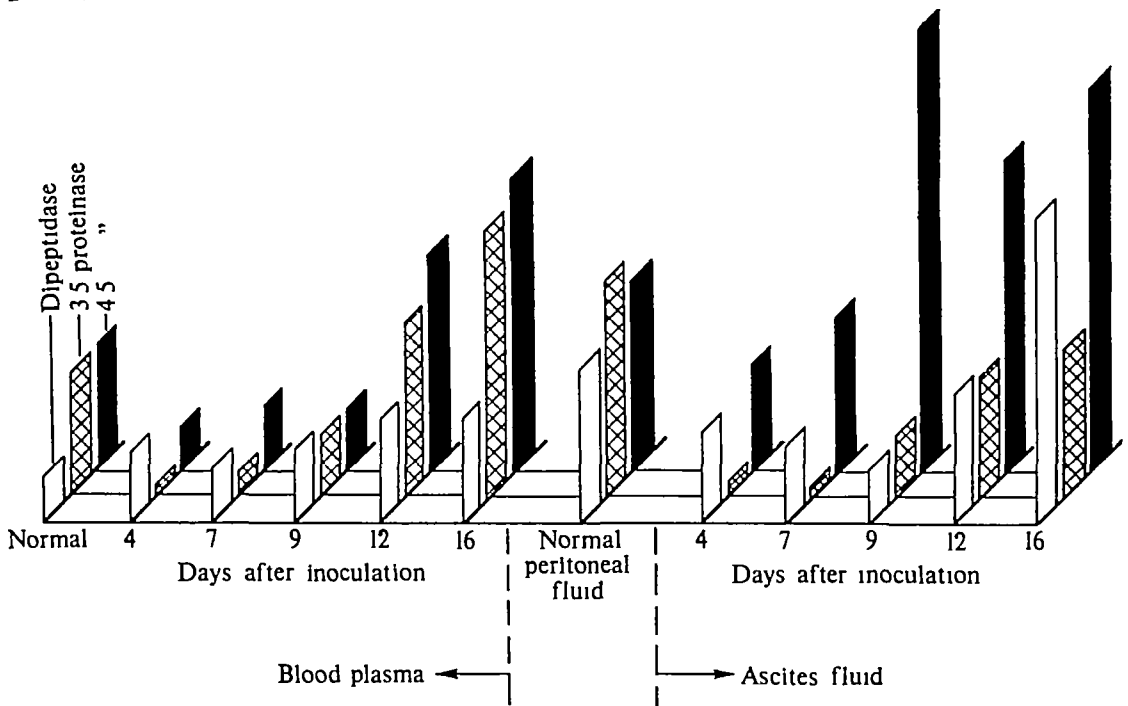

Fig 7 —Comparison between the enzymal activities expressed per μl fluid in the fluid compartments under question The plasma data (Ottoson and Sylvén, 1959) are obtained on blood samples from the same mice as used in this report The data on normal peritoneal fluid will be published elsewhere

The relative enzymic activities of the fluid compartments under question are for comparison compiled in Fig 7 According to previous concepts it may be tentatively assumed that the labile extracellular dipeptidase activity is associated with a relatively small protein molecule of a size close to the albumins, while the pH 4 5 proteinase activity most likely resides in a larger molecule possibly of the size of the globulins

The dipeptidase concentration

In the course of the ascites tumour growth the plasma concentration of total dipeptidase activity increased to about twice the normal level (Ottoson and Sylvén, 1959) In the same mice the peptidase activity of the ascites fluid at first, before the 12th day after inoculation, had a similar concentration (Table II), but then rose to a maximum level of about three times that of the corresponding blood plasma activity, and twice that of the normal intraperitoneal fluid (Fig 7)

The ascites tumour cells had a very high content of peptidases The results can
be explained by the assumption that peptidases are diffusing from the tumour
cells into the ascitic fluid and then transported to the blood The relative balance
between the ascites fluid and blood plasma concentration during the first part
of tumour growth might signify that the exchange is more rapid during this time
(cf Berson and Yalow, 1954 , Straube, 1958) while possibly a relative retention
of peptidases becomes more evident later on Independent *in vitro* data (Sylvén,
unpublished results) clearly show that the tumour cells are releasing peptidases
at a high rate

The concentration of acid proteinases

The proteinase pattern presents a complicated picture During the first part
of the ascites tumour growth most of the acid proteinase activity was abolished
both in the blood plasma (Ottoson and Sylvén, 1959) and in the ascites fluid
(Fig 7) It is suggested that this may be due to an increased concentration of
acid serum mucoproteins acting as proteinase inhibitiors (cf above) The pH
3 5 enzyme certainly has some source other than the ascites tumour cells, since the
latter do not contain this enzyme (Fig 4) The enzyme on the other hand is
present in normal peritoneal fluid (unpublished results), although its actual
activity level there is lower than in normal mouse plasma It is possible that the
pH 3 5 enzyme is derived from the stomach mucosa according to Mirsky *et al*
(1952) The present data do not give reliable information as to the actual
concentrations of this enzyme due to the apparent presence of an inhibitor, and
therefore, further discussion is postponed

The pH 4 5 proteinase is present in the ascites tumour cells (Fig 4) Also this
enzyme seems to be influenced to some extent by the proteinase inhibitor in the
ascites fluid during the first 5 to 7 days of tumour growth In some series of mice
the pH 4 5 enzyme shows a moderate activity in the ascites fluid at the 5th to
7th day after inoculation (Fig 5) The activity then increases to a high level in
the blood plasma as well as in the ascites fluid, although the activity in the latter
compartment remains somewhat higher On the assumption that the inhibitor
concentration is of the same magnitude in both compartments, it appears probable
that a transport of this enzyme occurs from the cells via the ascites fluid to the
blood

The fate of the peptidases and proteinases possibly transferred to the blood
stream is not known , a certain degree of inactivation and/or excretion is assumed
to occur The peptidases are for instance extremely sensitive, and may as well
as the proteinases become excreted similar to its pepsin counterpart The relative
ratio in plasma between the extinctions of the pH 3 5 and the 4 5 proteinase
activity leaves no indication as to the fate of the pH 4 5 plasma enzyme during
the later part of tumour growth

It will be noted that the observed differences in enzymic activities per volume
would become increased if the data are recalculated per protein content of the
different compartments (Table III) Since this may not be fully justified with a
view to the transport problems involved, the authors have omitted to consider
this set of data in detail

The present results do not allow us to consider to what extent a selective
retention of enzymes might contribute to the observed increases of enzymic

activity in the ascites compartment The determinations are only an indication of the actual activities Since uncontrolled factors are involved and in particular the presence of a proteinase inhibitor is assumed, the observed activity data do not represent the total enzyme contents, and cannot serve as indicators of the amount of blood proteins filtering through from the serum Present data are therefore only circumstantial evidence in favour of the view that tumour cells, as well as normal cells, release enzymes to different extents into the extracellular fluid

The reported *in vivo* experiments, however, are insufficient to explain the factors and environmental conditions responsible for the marked enzymic increase at the end of the ascites tumour growth It is not known whether this increase is associated with an increased rate of cell death on the part of the tumour cells Ács and Straub (1954) described a very marked increase in the concentration of potassium ions in the ascites tumour fluid This can also be interpreted as an indication of a release of intracellular K ions into the extracellular space The increase in the K ion concentration was noted already very early during tumour growth, and did not seem to have a close correlation with the frequency of cell death

The report illustrates the difficulties involved in the interpretation of data derived under *in vivo* conditions, where large differences in enzymic activity are required before significant results can be obtained on a per-volume basis

SUMMARY

Quantitative assays were performed of the dipeptidase activity, against alanylglycine, and proteinase activity between pH 3 to 6, using urea-denatured haemoglobin as a substrate, in the cell-free ascites fluid of inbred male mice inoculated with a standard dose of the hyperdiploid Ehrlich ascites tumour Comparison was made with previous serial assays, performed in the same mice, on the corresponding activities of blood plasma

The quantitative growth characteristics of this ascites tumour line are described The protein concentration of the cell-free ascites fluid descreased to a figure of about 2 5 per cent at the end of tumour growth The changes in both the absolute and relative distribution of albumins and globulins in the blood plasma and ascites fluid of tumour-bearing mice were determined by means of electrophoresis

The dipeptidase activity of the ascites fluid showed a continuous increase in the course of tumour growth, and reached shortly before the animals died a level of about three times that observed in the corresponding blood plasma The pH 3 5 and 4 5 proteinases, normally present in blood plasma, were also demonstrable in the normal extracellular peritoneal fluid The pH 3 5 proteinase activity was almost abolished in the early ascites fluid as well as in the plasma of the same mice The pH 4 5 activity was simultaneously somewhat depressed The pH 3 5 proteinase activity later on reappeared in both compartments, but only reached a level of about half the activity of the corresponding blood plasma The pH 4 5 proteinase activity is similar to the intracellular cathepsins This reached a high level in the ascites fluid constituting its main proteinase in the acid pH range During the same time this activity was increased to twice the normal level in the blood plasma

The conclusion is reached that, most likely, the increase in dipeptidase and pH 4 5 proteinase activity in the ascites fluid originate by release of enzymal proteins from intraperitoneal cells   Other independent data to be reported elsewhere support the view that most of the released enzymes have leaked out from the tumour cells

The results are so far only valid in the case of ascites tumours , the plasma activities in mice with solid transplanted tumours remain to be studied

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MITOCHONDRIA OF THE EHRLICH ASCITES TUMOUR CELL —SWELLING CHARACTERISTICS

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THE contention of Warburg (1956) that tumour cell mitochondria are irreparably damaged, has prompted numerous comparisons between the properties of normal and tumour cell mitochondria   The isolation of Ehrlich ascites tumour mitochondria and studies of their oxidative phosphorylation with a variety of substrates were reported in a previous communication from these laboratories (Hawtrey and Silk, 1959)   The present report concerns further studies, but of a physical nature—namely, the swelling characteristics of both respiring and non-respiring Ehrlich ascites mitochondria in a variety of media   The characteristics have been compared with those of mitochondria from normal cells and from other tumour types under similar conditions

A comparison between the swelling characteristics of normal and tumour cell mitochondria provides information concerning the influence of intracellular and extracellular fluid environment on mitochondrial functioning   Studies in these laboratories have demonstrated that the fluid environment of tumour cells is of paramount importance as the medium through which X-radiation acts " indirectly " on respiratory metabolism (Silk, Hawtrey and Macintosh, 1958)   The environmental " indirect " action of X-radiation is at present being studied using Ehrlich tumour mitochondria as test material in place of whole cells   Accordingly it became necessary to investigate the influence of environmental medium pH and composition on the physical characteristics of Ehrlich tumour mitochondria   Such physical studies are of importance because of the possible influence of swelling and shrinkage on the structural arrangement of enzymes necessary for chemical functioning of the mitochondria (Tedeschi and Harris, 1958)

MATERIALS AND METHODS

Ehrlich ascites tumour —This tumour was kindly supplied by Dr K Sugiura of the Sloan-Kettering Institute, New York, and has been maintained in Swiss albino mice which were used between the 9th and 12th day after inoculation

Hexokinase (General Biochemicals Inc , Ohio, U S A) when assayed according to Somogyi (1952) and Crane and Sols (1955) was found to have an activity of approximately 150 units per mg

Cytochrome c was used as a 1 per cent solution in isotonic saline as supplied by General Biochemicals Inc , and was found to contain $5\ 87 \times 10^{-7}$ moles per ml when assayed according to Umbreit, Burris and Stauffer (1957)

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Adenosine-5-phosphoric acid (*AMP*) and *Adenosine diphosphate* (*ADP*) were used as supplied by General Biochemicals Inc, and by the H M Chemical Co of Los Angeles, California respectively

Adenosine triphosphate (*ATP*) was used as the tetra sodium salt supplied by General Biochemicals Inc, and assayed as 85 per cent pure by the method of Crane and Sols (1955)

Diphosphopyridine nucleotide (*DPN*) was obtained from General Biochemicals Inc, and assayed as 85 per cent pure by a modification of the method of Brodie (1955) using 10 mg of sodium dithionite per 1 0 ml of 0 2 M dibasic sodium phosphate as the reducing solution

All other substrates and chemicals used were of Analytical Reagent grade

Isolation of mitochondria

Mitochondria were isolated by the method of Hawtrey and Silk (1959)   All operations were carried out at 0–4° C   Saline washed ascites cells were allowed to swell for 15–20 minutes in hypotonic ($\frac{1}{4}$ strength) Ringer phosphate solution, and subsequently disrupted into 8–10 volumes of pure water using a loose fitting Dounce homogeniser (Blaessig Glass Specialties, Rochester, New York, U S A) for exactly 3 0 minutes   Immediately after lysis, concentrated sucrose solution was added to the homogenate to give the usual 0 25 M sucrose suspension from which the mitochondria were isolated by differential centrifugation   Nuclei and cell debris were removed at 600–700 g for 20 minutes, and the supernatant centrifuged at 12,000–13 000 g for 10 minutes to sediment the mitochondrial fraction   The mitochondria were washed twice until free of " fluffy " layer, and were then re-suspended in 0 25 M sucrose (approximately 5–6 ml per g wet weight) equivalent to a concentration of 4–6 mg mitochondrial protein per ml   The pH of this final suspension was 6 9

Mitochondrial protein content was determined according to the biuret method of Cleland and Slater (1953) using an ovalbumin standard

Media for determination of mitochondrial swelling

(a) *Non-respiring mitochondria* —The medium is indicated for each experiment

(b) *Respiring mitochondria* —The following medium was used   0 045 M sodium potassium phosphate buffer (pH 7 4), 0 015 M KF, 0 008 M $MgCl_2$ 0 0025 M ADP, 0 001 M AMP, 0 002 M EDTA (ethylene diamine tetra acetic acid), 0 01 M d-glucose, 0 032 M sucrose, $2 4 \times 10^{-4}$ M DPN, $9 8 \times 10^{-6}$ M cytochrome c, 1–3 mg hexokinase (crystalline), 0 007 M substrate   (Media with α-ketoglutarate as substrate contained 0 02 M malonate in addition   AMP ADP and all substrates were neutralised to pH 7 4 with KOH and stored at −30° C before use)

For experiments in which co-factors or the glucose-hexokinase phosphate acceptor system were omitted from the medium, 0 25 M sucrose was used to make up the necessary volume

Measurement of mitochondrial swelling

The change in optical density of a dilute suspension of mitochondria was measured according to the method of Cleland (1952)

Measured volumes (0 15 ml) of the mitochondrial suspension in 0 25 M sucrose

(containing 4–6 mg protein/ml) were added to 2 85 ml of the medium in which swelling was to be determined   After thorough mixing, the optical density at 520 mμ was read in a Model DU 2400 Beckman Spectrophotometer against a blank containing the medium under examination plus 0 25 M sucrose in place of the mitochondrial suspension   Values are expressed as Specific Extinction coefficients

$$\left(E\ \frac{1\%}{1\ cm} \right)$$

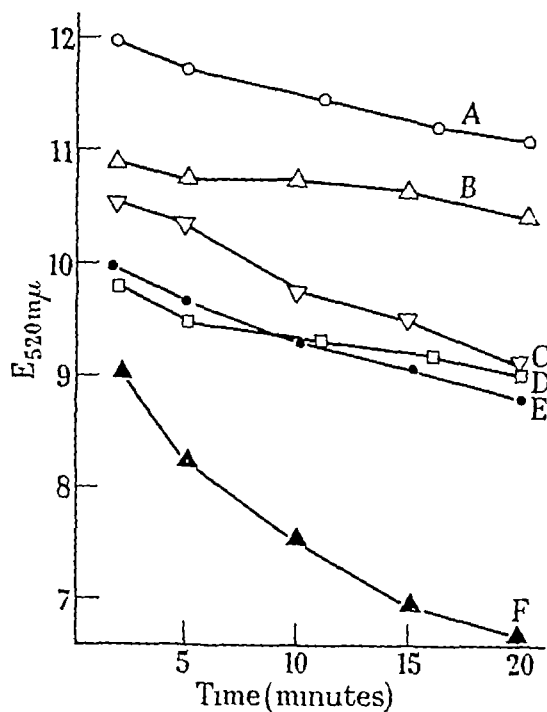for suspensions containing 1 per cent of mitochondrial protein
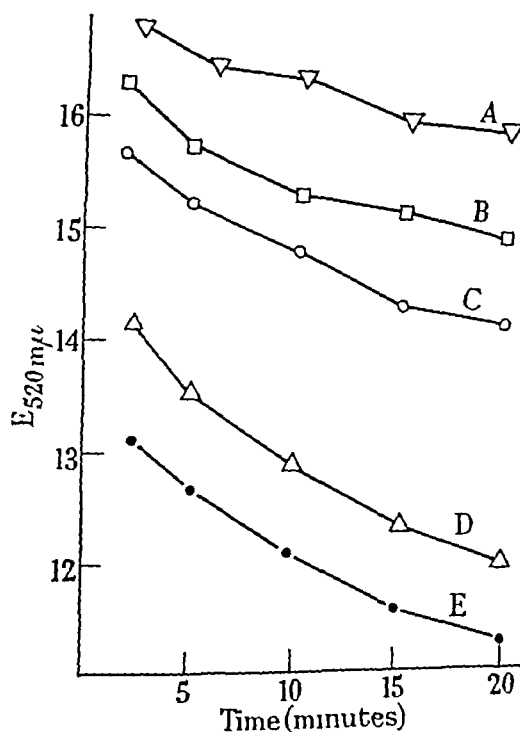

FIG 1   FIG 2

FIG 1 —Swelling of non respiring mitochondria in unbuffered sucrose solutions   A, 0 25 M sucrose + 0 01 M EDTA (pH 7 4) , B, 0 25 M sucrose , C, 0 44 M sucrose , D, distilled water , E, 0 80 M sucrose , F, 0 60 M sucrose

FIG 2 —Swelling of non respiring mitochondria in sucrose solutions buffered to pH 7 4 by addition of 0 05 M tris (hydroxymethyl) aminomethane acetate   A, 0 25 M sucrose + 0 01 M EDTA , B, 0 25 M sucrose , C, 0 44 M sucrose , D, 0 60 M sucrose , E, 0 80 M sucrose

RESULTS

Each family of curves shown in Fig 1–9 was obtained using a single mitochondrial preparation   A control determination of Q_{O_2} value and P O ratio with succinate as substrate was carried out on each batch of mitochondria, to ensure their functional integrity for use in swelling experiments   All the preparations used showed a satisfactory level of oxidative phosphorylation activity with succinate (cf Hawtrey and Silk, 1959) Q_{O_2} values lay within the range 85–125 and P O ratios within the range 1 1–1 4   (Q_{O_2} values are expressed as μl O₂ uptake per mg protein per hour)

Swelling of non-respiring mitochondria

The curves in Fig 1 show that in unbuffered solutions the swelling of non-respiring mitochondria is minimal in 0 25 M sucrose which was used in the isolation procedure   Swelling of mitochondria was also negligible in water which was used as suspending medium for initial lysis of the ascites cells   No agglutination of mitochondria was observed in aqueous suspension   A certain amount of mitochondrial swelling was observed in sucrose solutions above 0 25 M in concentration   The presence of 0 01 M EDTA caused a slight increase in the swelling observed in 0 25 M sucrose   The swelling of non-respiring mitochondria in sucrose solutions buffered to pH 7 4 with tris-(hydroxymethyl)-aminomethane-acetate (Fig 2) was on the whole slightly more than in unbuffered solutions (Fig 1)   As with
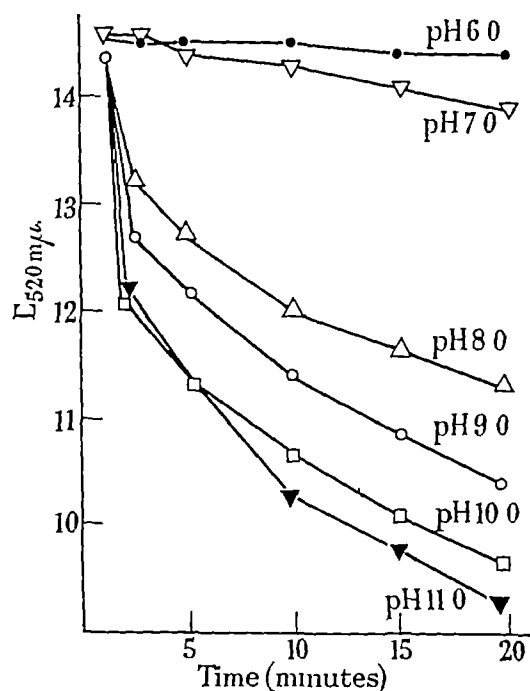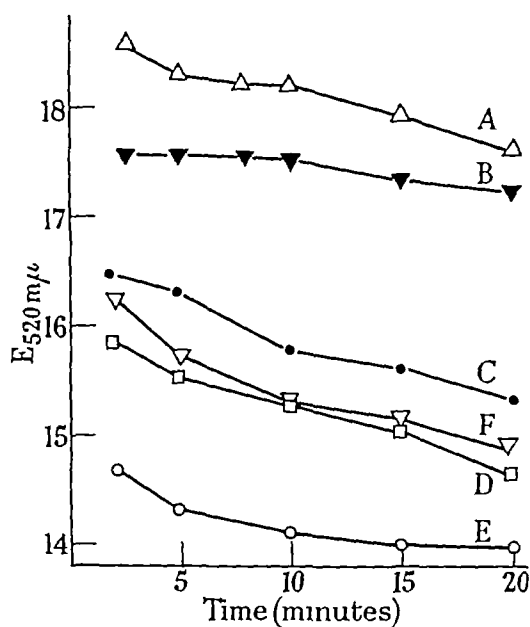

FIG 3

FIG 4

FIG 3 —The effect of pH on the swelling of non respiring mitochondria   Mitochondrial suspension in 0 25 M sucrose (0 15 ml) was added to 2 85 ml of 0 25 M sucrose buffered with 0 05 M tris acetate to the pH indicated for each curve   At pH values below 6 0, agglutination of the mitochondria occurred

FIG 4 —Swelling of non respiring mitochondria in 0 25 M sucrose solutions buffered with 0 05 M tris acetate (pH 7 4), and containing added co factors   A, 0 001 M ATP , B 0 001 M DPN , c, 0 001 M AMP , D, 0 001 M ATP + 0 001 M DPN , E, 0 001 M ADP , F, control

unbuffered solutions the swelling was least in 0 25 M sucrose, but the increase of swelling on addition of EDTA to unbuffered solutions was not observed in buffered solutions   Whereas with unbuffered solutions, swelling was greatest in 0 60 M sucrose and less in 0 80 M (Fig 1), in buffered solutions swelling was comparable in both concentrations of sucrose (Fig 2)

Non-respiring mitochondria did not swell in sucrose solution buffered to pH 6 0 with tris-acetate, but swelling was observed at pH 7 0 and was found to be

increased at successively higher pH values (Fig 3)   Agglutination of the mitochondria occurred below pH 6 0

The swelling of non-respiring mitochondria in 0 25 M sucrose buffered to pH 7 4 with tris-acetate was considerably decreased by addition of 0 001 M DPN to the medium (Fig 4)   Addition of ADP caused a slight inhibition of swelling, while AMP and ATP were without effect   The protective action of DPN was nullified when ATP was also present in solution (Fig 4)
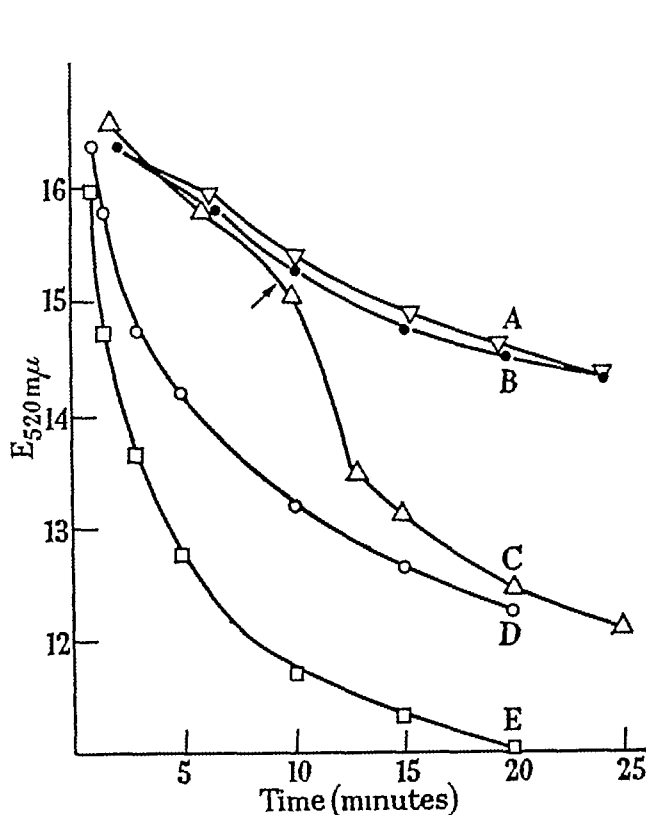

FIG 5 —Swelling of non respiring mitochondria in 0 25 M sucrose solutions buffered with 0 05 M tris acetate (pH 7 4) and containing various additions   A, 1 2 × 10⁻⁴ M 2 4-dinitrophenol (DNP), B, control, c, 3 × 10⁻⁵ M L thyroxine added as indicated (↗), D, 3 × 10⁻⁵ M L-thyroxine, E, 0 01 M K, Na phosphate + 0 001 M potassium succinate
FIG 6 —Swelling of respiring mitochondria in phosphorylating medium (see text) with various substrates   A, 0 01 M succinate, B, 0 0066 M ascorbate, c, 0 01 M DL β hydroxybutyrate, D, 0 0066 M L glutamate, E, no substrate
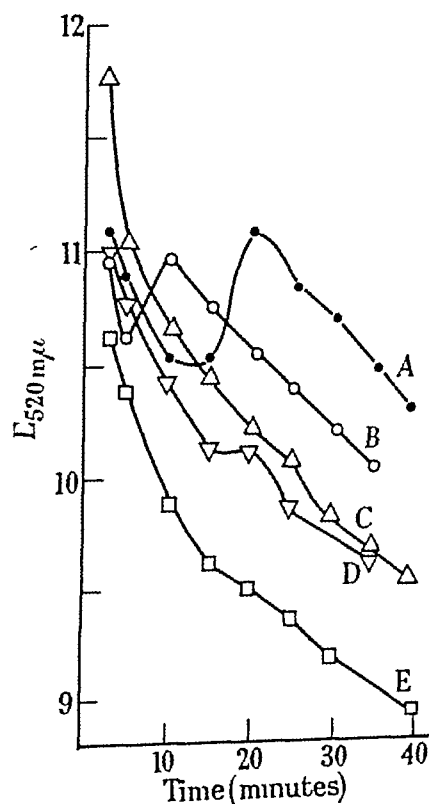
The swelling of non-respiring mitochondria in 0 25 M sucrose buffered to pH 7 4 with tris-acetate was not affected by addition of the uncoupling agent DNP (2 4 dinitrophenol)   On the other hand addition of thyroxine *ab initio* or at a subsequent stage in the experiment, caused a marked increase in swelling (Fig 5) but only with 6 out of 8 mitochondrial preparations examined   Rapid swelling of non-respiring mitochondria in buffered sucrose was observed on addition of succinate and phosphate to the medium (Fig 5)

Swelling of respiring mitochondria

Mitochondria in the phosphorylating medium without added substrate, underwent the same degree of swelling as observed for non-respiring mitochondria

in 0 25 M sucrose   However, in the presence of certain added substrates shrinkage occurred followed by a gradual degree of swelling (Fig 6)   Succinate caused the maximum shrinkage of swollen mitochondria, while ascorbate was practically as effective though more rapid in its action   Addition of L-glutamate caused only mild shrinkage of the mitochondria while addition of β-hydroxybutyrate was without effect on the swelling (Fig 6)
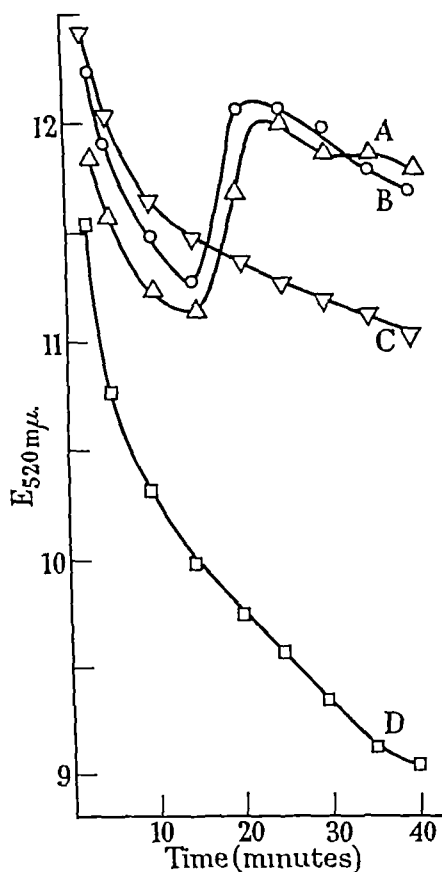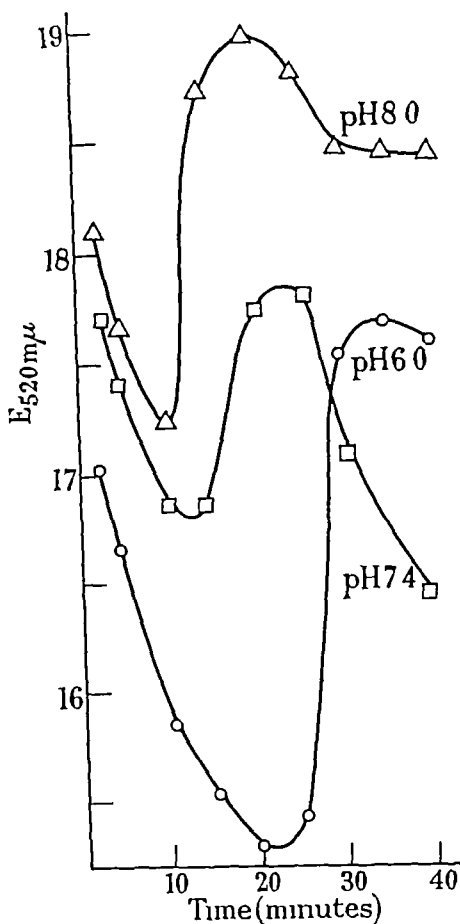
FIG 7   FIG 8

Fig 7 —Swelling of respiring mitochondria in phosphorylating medium (see text) with succinate as substrate   Effect of removing co factors from the medium   A, no hexokinase , B, control , C, no cytochrome c or hexokinase , D, no cytochrome c

FIG 8 —Swelling of respiring mitochondria in phosphorylating media at various pH values with succinate as substrate   pH 8 0   medium as in text but with (0 005 M K,Na phosphate buffer + 0 040 M tris-acetate buffer) in place of the 0 045 M K,Na phosphate buffer specified   pH 7 4   medium as in text   pH 6 5   medium as specified for pH 8 0   Agglutination of mitochondria occurred below pH 6 0, and heavy precipitates formed in the medium above pH 8 0

The shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was unchanged by removal of the hexokinase-phosphate esterifying system from the medium (Fig 7)   On the other hand removal of cytochrome c from the medium resulted in rapid swelling and complete failure

of the mitochondria to undergo shrinkage Removal of cytochrome c and hexokinase together also resulted in failure to undergo shrinkage, but the degree of initial mitochrondrial swelling was less than in the case where only cytochrome c was removed from the medium (Fig 7)

The effect of medium pH on the shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was studied in a series of experiments Shrinkage took place after 10 minutes at pH 8 0, after 12 minutes at pH 7 4 but only after 22 minutes at pH 6 0 Agglutination of mitochondria occurred below pH 6 0, while heavy precipitates formed in the medium above pH 8 0 (Fig 8)
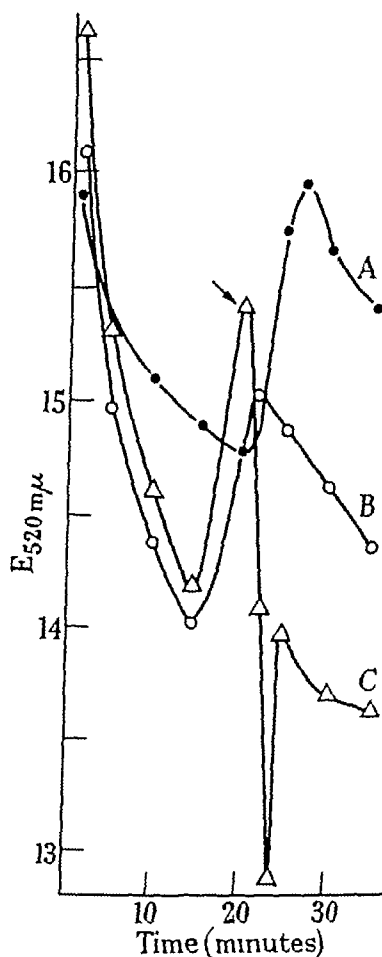
FIG 9 —Effect of thyroxine on swelling of respiring mitochondria in phosphorylating medium (see text) with succinate as substrate A, 3×10^{-5} M thyroxine present initially, B, no thyroxine, C, 3×10^{-5} M thyroxine added as indicated ($\times$)

Shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was also observed in the presence of 3×10^{-5} M thyroxine, although the initial swelling was less than in a medium without thyroxine Addition of 3×10^{-5} M thyroxine to the medium immediately after shrinkage of the respiring mitochondria had taken place, caused instantaneous and marked swelling followed by rapid shrinkage and a subsequently more gradual rate of swelling (Fig 9)

DISCUSSION

Swelling of non-respiring Ehrlich tumour mitochondria was minimal in water and in 0 25 M sucrose used for the isolation procedure. The method of isolation involved brief contact of the mitochondria with water over a period of 3 0 minutes during initial lysis of the ascites cells, but it has been shown that such treatment does not impair their capacity for oxidative phosphorylation (Hawtrey and Silk, 1959). Concentrated sucrose solution is added to the homogenate immediately after the 3 0 minute lysis period and thus, in all probability, completely reverses any swelling which may occur during contact with the water (Tedeschi and Harris, 1958).

The observation that initial $E_{520 m\mu}$ values for a single batch of mitochondria are different in sucrose solutions of varying osmolarity (cf Fig 1 and 2) is attributable to differences in refractive index of the suspending media (Tedeschi and Harris, 1958). The same divergence of initial $E_{520 m\mu}$ values in varying sucrose concentrations was found by Mutolo and Abrignani (1957). In a later paper by Mutolo and Abrignani (1958) curves are shown for tumour mitochondria which have the same initial $E_{520 m\mu}$ value for a single sucrose concentration. Our results also show that in a single medium the swelling curves for Ehrlich mitochondria tend towards a common origin.

The observation that non-respiring tumour mitochondria show an increased degree of swelling with increasing sucrose molarity in buffered or unbuffered solution, is in contrast to the finding of Lehninger (1956) that rat liver mitochondria exhibit least swelling in hypertonic sucrose. The apparent increase of swelling with increasing solution osmolarity is in itself not easily explicable, but may be due to a gradual change in internal refractive index of the mitochondrial particles (cf Tedeschi and Harris, 1958, and Barer, Ross and Tkaczyk, 1953).

Failure of EDTA to prevent the swelling of non-respiring Ehrlich mitochondria in unbuffered sucrose is in keeping with the results of Mutolo and Abrignani (1957) who found that it tended to promote the swelling of Walker 256 carcinosarcoma mitochondria. EDTA effectively prevents the swelling of rat liver mitochondria (Mutolo and Abrignani, 1957), and also stabilises the oxidative activity of heart muscle sarcosomes (Cleland and Slater, 1952).

The increase in swelling of non-respiring Ehrlich mitochondria in 0 25 M sucrose at successively higher pH values, parallels the results of Witter and Cottone (1956) using rat liver mitochondria. Liver and Ehrlich tumour mitochondria show least swelling at pH 6 0, and below pH 6 0 rapid agglutination of both types occurs (Gamble, 1957).

The swelling of non-respiring Ehrlich mitochondria in 0 25 M sucrose buffered to pH 7 4 was not reversed on addition of ATP, ADP, AMP, DPN or ATP + DPN to the medium. DPN and to a lesser extent ADP were however effective in decreasing the rate of swelling, although the protective action of DPN was nullified when ATP was also present in solution. By contrast Fonnesu and Davies (1956) and Dickens and Salmony (1956) found the swelling of liver mitochondria to be reversed by addition of ATP. A similar effect was shown by Chappell and Perry (1954) using heart muscle sarcosomes. Failure of ATP and DPN to reverse the swelling of Ehrlich ascites cell mitochondria, may be due to the high ATP-ase and DPN-ase activities of tumour mitochondria as distinct from those of normal

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cells No fluoride or nicotinamide were added to inhibit these actions in the media used to examine the Ehrlich mitochondria

The uncoupling agent 2 4 dinitrophenol (DNP) was without effect on the swelling of non-respiring Ehrlich mitochondria in 0 25 M sucrose buffered to pH 7 4 On the other hand, Lehninger (1956) observed that DNP effectively prevented the swelling of rat liver mitochondria Thyroxine, another uncoupling agent, caused a rapid increase in the swelling of non-respiring Ehrlich mitochondria which parallels the findings of Dickens and Salmony (1956) and Lehninger (1956) with non-respiring rat liver mitochondria, and is in contrast to the findings of Emmelot and Bos (1957) with rat hepatoma mitochondria However, the effect of thyroxine on normal cell mitochondria is apparently variable Tapley and Cooper (1956) found that rat liver and kidney mitochondria underwent swelling in the presence of thyroxine whereas those of the heart, testis and spleen did not

Rapid swelling of non-respiring Ehrlich tumour mitochondria was observed in the presence of oxidizable substrate (succinate) and inorganic phosphate This is analogous to the behaviour of rat liver mitochondria reported by Chappell and Greville (1958), and is in keeping with the statement of Witter and Cottone (1956) that succinate and phosphate cause a breakdown of organised mitochondrial structure In a fortified phosphate medium but without substrate, Ehrlich mitochondria underwent the same degree of swelling as observed in 0 25 M sucrose buffered to pH 7 4

The swelling of respiring Ehrlich mitochondria was studied in the same fortified incubation medium with a variety of substrates Uninterrupted swelling was observed with L-glutamate and β-hydroxybutyrate, possibly due to the low quantity of DPN present, but with succinate and ascorbate an apparent reversal of swelling was observed Moreover, this reversal occurred only in the presence of added cytochrome c and was unaffected by removal of the hexokinase trapping system The possibility exists that two phenomena are involved simultaneously in the observation of swelling reversal at 520 mμ, (a) actual physical shrinkage of the mitochondria in the presence of cytochrome c, and (b) reduction of the external cytochrome c which could act as an electron acceptor if the conditions in the Beckman cuvette were anaerobic at the time when the reversal of swelling was observed Part of the increased absorption at 520 mμ could therefore be due to the β-band of reduced cytochrome c Blank experiments in which solid sodium dithionite was added to the cuvette at the start of the observed shrinkage, showed only 30 per cent of the increase in optical density usually found with succinate as substrate Thus 30 per cent of the increase in optical density observed after 15 minutes with succinate and ascorbate may be due to reduction of the external cytochrome c, while 60 per cent may be due to actual shrinkage Holton (1955) and Chance (1957) have shown that reduction of cytochromes in a mitochondrial suspension can be followed in the Beckman spectrophotometer

Shrinkage of respiring mitochondria in the presence of succinate and cytochrome c was found to take place more rapidly at successively higher values of pH Agglutination of the mitochondria occurred below pH 6 0 and above pH 8 0 heavy precipitates formed in the solution

If added initially thyroxine decreased the usual rate of swelling of respiring Ehrlich mitochondria, whereas the opposite effect was observed with non-respiring

mitochondria   However, if thyroxine was added when maximum shrinkage of respiring mitochondria had taken place, an immediate swelling of the particulates was observed   This was followed first by rapid shrinkage and then by a more gradual rate of swelling

The physical characteristics of Ehrlich ascites cell mitochondria therefore appear to be similar to those reported for other tumour mitochondria, but differ in a number of respects from those of normal cell mitochondria   However, on the basis of the comparisons drawn it would not be possible to decide whether the observed differences might be generally characteristic of tumour cell mitochondria

SUMMARY

1 The swelling characteristics of both respiring and non-respiring Ehrlich ascites cell mitochondria have been examined in a variety of media

2 Swelling of non-respiring mitochondria is minimal in water and in 0 25 M sucrose and is not inhibited by addition of versene to the medium

3 Swelling of non-respiring mitochondria increased with successive increases in pH value of the suspending medium   Agglutination of the mitochondria occurs below pH 6 0

4 The swelling of non-respiring mitochondria cannot be reversed by addition of ATP, ADP, AMP, DPN or ATP + DPN to the medium   However, DPN and, to a lesser extent, ADP are effective in decreasing the rate of swelling

5 Dinitrophenol is without effect on the swelling of non-respiring mitochondria in buffered sucrose   Thyroxine causes a rapid increase in swelling of non-respiring mitochondria

6 Rapid swelling of non-respiring mitochondria occurs in the presence of oxidizable substrate (succinate) and inorganic phosphate

7 Mitochondria allowed to respire with ascorbate and succinate as substrates apparently undergo a temporary reversal of swelling in the presence of added cytochrome c   The phenomenon is not observed with L-glutamate and β-hydroxy-butyrate as substrates, possibly due to the low quantity of DPN present in the test medium

8 The apparent reversal of mitochondrial swelling observed with succinate and ascorbate as substrates may be due in part to actual shrinkage and in part to reduction of external cytochrome c

9 Shrinkage of respiring mitochondria in the presence of succinate and cytochrome c takes place more rapidly at higher values of medium pH   As with non-respiring mitochondria, agglutination occurs below pH 6 0

10 Thyroxine added initially to respiring mitochondria decreases the usual rate of swelling   If added when maximum shrinkage of swollen mitochondria has occurred, thryoxine causes an immediate swelling followed by rapid shrinkage   This is followed again by a more gradual rate of swelling

11 The swelling characteristics of Ehrlich tumour cell mitochondria have been compared with those reported for mitochondria from other tumour types and from normal cells

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THE OCCURRENCE OF CANCER IN HUSBANDS AND WIVES

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THIS investigation was undertaken as an attempt to answer the question " How important are *common* domestic environmental factors in the aetiology of cancers? "

There is a large literature devoted to studies of cancer in families In these studies the influence of genetic and environmental factors are usually almost inextricably interwoven   (An exception to this generality is the study of cancer occurring in twins separated at birth, but, even here, we have a common ante-natal environment which is of at least theoretical importance)

As we cannot conveniently remove environmental factors in studies of family or household cancer, one must consider how genetic factors might be eliminated, leaving only those of environment   By genetic factors here we refer to inherited factors shared by members of the same family rather than to genetic factors common to a community   This means that we have to study the occurrence of cancer in members of the same households who are not blood relatives   Material for such a study would be found by examining the causes of death of husbands and their wives   This is the approach used in the present investigation

Hypotheses tested in the investigation

If one made the hypothesis that gastric carcinomata often had their causation in the consumption of " over-cracked " cooking fats, a deduction might be made which could be tested by observation   Dr Percy Stocks has pointed out (personal communication) that, very likely, even cancer of a particular site may be produced by various causes   This deduction might run—" Husbands and wives tend to eat food that has been cooked by a common method X   Therefore, if cooking habit X (e g cracked fat) is aetiologic in many cases of stomach cancer, one would expect to find that, where wives had this disease, their husbands would tend to be at statistical risk of suffering from it also, eventually "   The same argument, in a broader or more general form, would run thus—" Whatever factors a b c   in the domestic environment, common to husband and wife, that cause cancer in one partner, may be expected, when other factors are equal, to produce cancer in the other partner "   As a corollary to this one could formulate   " If cancer generally (or specific cancers) tend to be associated in husband and wife

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more than would be expected on the basis of chance (as determined by controlled studies) it is likely that there is a common domestic environmental factor (or factors) operative in the aetiology of their neoplasms " Conversely, to formulate the negative proposition, one might assert it as likely that " If cancer in the husbands of women who had died of cancer is no commoner than cancer in the husbands of women who did not die from cancer, then it is unlikely that common domestic factors are important carcinogenic agents "

These hypothetical considerations were put to the test of observation in the manner now described

MATERIAL

The study was made from death certificates Copies of these were available for the following districts for years given in brackets —

City of Winchester, 18 years (1939 to 1956 inclusive)
Urban District of Merton and Morden, 18 years (1939 to 1956 inclusive)
Borough of Eastleigh, 19 years (1938 to 1956 inclusive)
Urban District of Banstead, 19 years (1938 to 1956 inclusive)
Municipal Borough of Gosport, 3 years (1954 to 1956 inclusive)
Metropolitan Borough of Fulham, 20 years (1937 to 1956 inclusive)
Deaths of residents dying both in and outside the districts were used

METHOD AND PROCEDURE

Every death certificate of a female in the above towns during the period (in most places fifteen to twenty years) was examined, and all widows dying from cancer, who totalled 1869, had small cards prepared giving name, date of death age, diagnosis in international classification of causes of death, serial number, address, husband's Christian names and occupation

1869 Control widows were then selected from among those who died of non-neoplastic conditions according to the following criteria Each cancer widow (the words " cancer widow " mean a widow who herself died from cancer) was matched with a control widow (a) who died during the same year, if possible in the same month, or at least the same quarter (b) who died at the same age exactly, or at least within two years of the same age (c) who came from the same district

Exceptions to these rules were extremely few, and occurred when either (i) the number of deaths in the district under survey was small, or (ii) the age on death of the " cancer " widow was so low that a " control " widow could not be found

The same facts as were noted for the cancer widows were noted for the control widows on similar cards Much care was taken in selecting the " controls ", and, subject to the factors mentioned in (i) and (ii), the highest degree of adherence to rules (a) and (b) was obtained by a general review of widows dying from neoplastic conditions, to see the possibilities of accurate matching, before selection for matching took place

After a final re-check of matching pairs in respect of age and date of death, the two sets of cards were inserted in racks, in alphabetical order, commencing with 1956, and, as each year's cards were racked, the death records for that year were

read aloud by one worker, while the other worker scanned the racks in the following way   The surname of each male entry whose age at death was consistent with his having been married before death (i e approximately 18 and over) was read out   When a surname was read which occurred among the cards " racked ", the husband's Christian names and occupation and the addresses of both widow and male entry were compared , where all three tallied it was assumed that the two entries were husband and wife , comparison of ages at year of husband's death was used as a final check, and if all factors pointed to a reasonable presumption of " husband " and " wife ", the following particulars of the husband were entered on the reverse of the widow's card   Date of death, age, diagnosis and serial number   Cases occurred in which one or more of the necessary factors were not known in either the widow's or husband's case, e g in Banstead the only address of the one partner was often given as Banstead Mental Hospital , in such cases, if the Christian names and occupation of the male entry were comparable on both card and entry, and age at death was also consistent with marriage, then the same assumption was made as when addresses also tallied   If husband's occupation was not given on either card or entry, Christian names and age were taken as basis of a reasonable assumption of marriage , but if neither Christian names nor occupation were given in both cases, so that at least one of the two factors could be compared, age was not considered sufficient basis for any assumption of marriage, and no action was taken

The search for the husbands of the 1869 widows who died from cancer revealed 417 pairs (i e husband and wife) in which the causes of death were known   When the search for the husbands of the 1869 " non-cancer widows " (i e widows who did not die from cancer) was completed, there was available a total of 455 pairs (each pair consisted of a widow who did not die from cancer and her husband, the cause of whose death was, again, known)

The information on the small cards was coded for cause of death in accordance with the 4 digit code of International Classification of Diseases   Occupation and social class were also coded according to the Registrar General's Classification of Occupations and Social Class Grouping   The cards were given pair numbers for matching husband and wife   Power Samas cards were then punched and verified for each of the 1744 individuals in the investigation to show death register entry number, place and date of death, sex, age, occupation (of husband) social class, cause of death (in parts 1 and 2 of the certificate) cause of death (in parts 1 and 2 of the certificate) of spouse, pair number and whether cancer widow or control widow or husband of cancer widow or husband of non-cancer widow

Comparability of final material

Although cancer widows and controls used in the original search were carefully matched, it was considered essential to examine the comparability of the final material   The purpose of this verification was to reduce the possibility that unknown selection factors had operated during the search for the husbands to upset the comparability of the two groups

Age comparability of cancer and non-cancer widows

Table I shows the numbers of cancer widows in five-year age groups, together with a column showing what percentage of all the 417 cancer widows is formed by those in each group   The table also shows the corresponding figures for non-

cancer widows, while Fig 1 shows the age distribution by these percentages for the two groups of widows It will be seen that the age composition of the two groups is closely comparable, so that failure to find the husbands of cancer widows of different ages has been paralleled by compensating failures in the search for the husbands of non-cancer widows in such a way that the matching of age groups has not been upset

TABLE I —*Age Composition of the Two Groups of Widows*

		40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	95–	All ages
Cancer widows	No	5	5	16	36	50	64	72	85	51	29	4	—	417
	%	1 2	1 2	3 8	8 6	12 0	15 4	17 3	20 3	12 2	7 0	1 0	—	100
Non-cancer widows	No	3	6	12	27	51	80	90	97	48	31	10	—	455
	%	0 7	1 3	2 6	5 9	11 2	17 6	19 8	21 3	10 6	6 8	2 2	—	100

FIG 1 —Age composition of the two groups of widows as percentages of each group (from Table I)

——————— Cancer widows 417
— — — — — — Non cancer widows 455

Social class comparability of cancer and non-cancer widows

Table II compares the social class distribution of the two groups of widows Fig 2 shows the percentage of these two groups in the different social classes from which it will be seen that the two groups were very similar in this respect

TABLE II —*Social Class Composition of the Two Groups of Widows*

		SOCIAL CLASS					Not stated	Total
		1	2	3	4	5		
Cancer widows	No	16	64	213	51	60	13	417
	%	3 8	15 4	51	12	14 5	3 3	100
Non cancer widows	No	21	68	235	65	58	8	455
	%	4 6	15	51 6	14 3	12 7	1 8	100

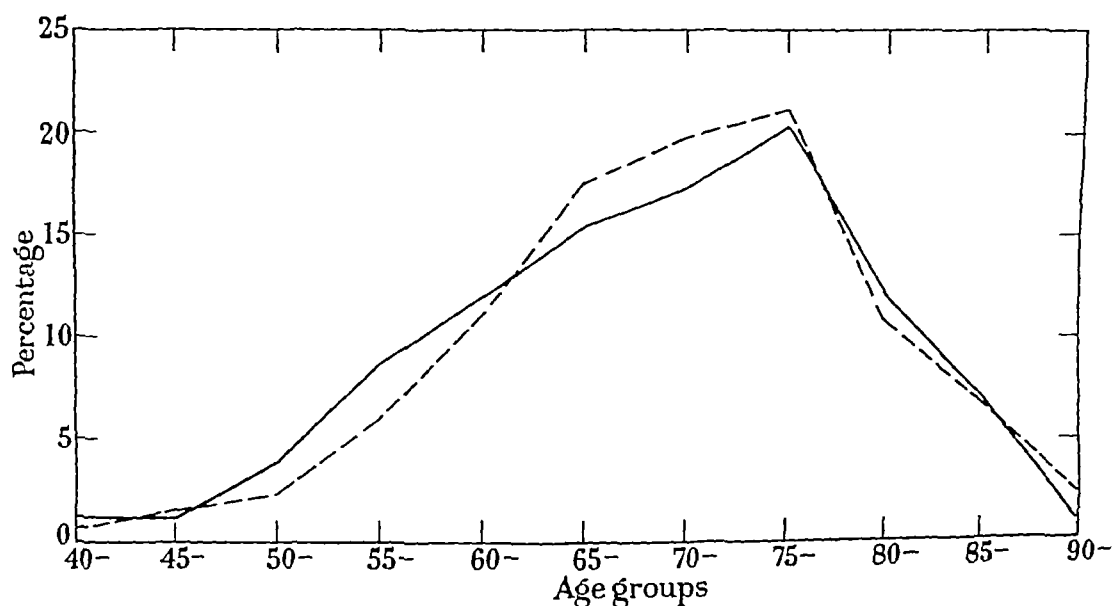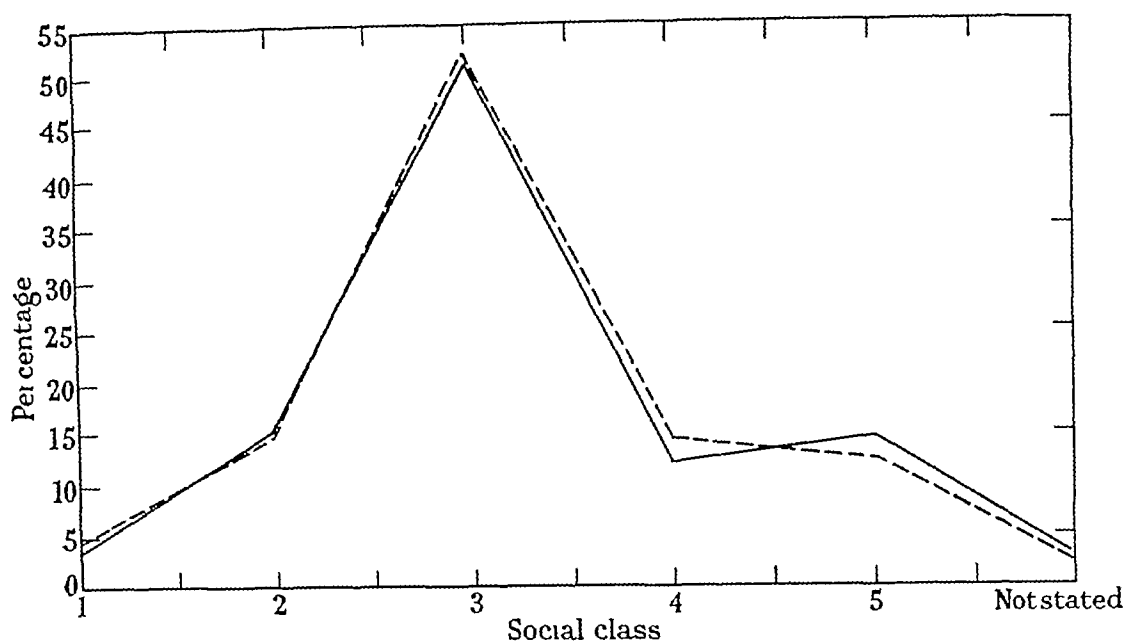FIG 2 —Social class composition of the two groups of widows as percentages of each group (from Table II)

——————— Cancer widows 417

— — — — — Non cancer widows 455

Comparability of cancer and non-cancer widows in respect to district

Table III and Fig 3 show that the proportion of cancer widows and non-cancer widows coming from different districts was closely comparable

TABLE III —*Places of Residence of the Two Groups of Widows*

		Gosport	Eastleigh	Win chester	Banstead	Merton and Morden	Fulham	All districts
Cancer widows	No	9	56	34	38	94	186	417
	%	2 2	13 4	8 3	9 1	22 5	44 5	100
Non cancer widows	No	13	63	32	27	109	211	455
	%	3 0	13 9	7 0	5 9	24 0	46 2	100

We conclude that we have then two groups of widows, one of which died from cancer, the other of which did not, which were comparable in respect of the age distribution of the group, social class, place of residence and year of death

The husbands—age composition

Table IV shows (columns 1 and 2) the age distribution (in five-year age groups) of the husbands of the cancer widows It also shows the percentage in each age group of the total of (417) husbands of cancer widows The corresponding figures for the 455 husbands of non-cancer widows is shown in columns 3 and 4 of the same table

Fig 3 —Percentage of each group of widows in the six districts

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– – – – – – – Cancer widows 417
——————— Non cancer widows 455
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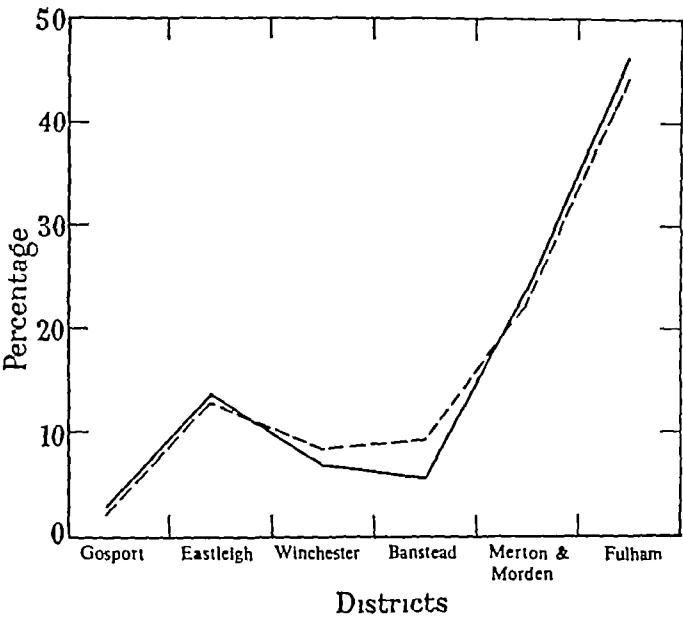Table IV —*Age Distribution of the Husbands of the Two Groups of Widows*

Husbands of Cancer Widows

Col No	30–	35–	40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	Not stated	All ages
1 No	—	—	5	12	26	39	52	77	89	56	50	9	2	—	417
2 %	—	—	1 2	2 9	6 2	9 3	12 6	18 4	21 3	13 4	12 0	2 2	0 5	—	100

Husbands of Non cancer Widows

Col No	30–	35–	40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	Not stated	All ages
3 No	1	2	4	6	25	28	65	86	83	86	47	13	8	1	455
4 %	0 2	0 4	0 9	1 3	5 5	6 2	14 3	18 9	18 2	18 9	10 3	2 9	1 8	0 2	100

The husbands—social class

The social classes of the husbands is, by definition, the same as that of their wives (see Table II and Fig 2)

The husbands—Geographical distribution

The district of residence of the husbands at the time of death is the same as that of their wives (see Table III and Fig 3)

RESULTS

1 *Cancer in the husbands of non-cancer (i e control) widows*

Of 455 husbands of non-cancer widows, 94 men died from cancer, i e 20 6 per cent

2 *Cancer in the husbands of cancer widows*

Of 417 husbands of cancer widows, 83 men died from cancer, i e 20 per cent

Further information about husbands dying from cancer

(a) *Age distribution*—Table V shows the age distribution of the 94 cancer deaths among the total of 455 dead husbands of non-cancer widows, together with the distribution of these deaths as percentages of the deaths in each age group of men who were husbands of non-cancer widows   It also shows the age distribution of the 83 cancer deaths among the total of 417 dead husbands of cancer widows, together with the distribution of these deaths as percentages of the deaths in each age group of men who were husbands of cancer widows

Table VI compares the age distribution of the cancer cases in the husbands of the cancer widows and the non-cancer widows   They are seen to be similar

TABLE V —*Age Distribution of Cancer Deaths Among the Two Groups of Husbands*

Age groups	Husbands of cancer widows			Husbands of non cancer widows		
	All husbands	Husbands who died from cancer	Per cent	All husbands	Husbands who died from cancer	Per cent
0–54	43	10	23 3	38	10	26 4
55–64	91	18	19 8	93	19	20 5
65–74	166	38	22 9	169	40	23 6
75+	117	17	14 5	155	25	16 2
All ages	417	83	20 0	455	94	20 6

TABLE VI —*Age Distribution of Cancer Cases in the Two Groups of Husbands*

Age groups	Husbands of cancer widows who themselves died of cancer		Husbands of non cancer widows who themselves died of cancer	
	Number	Per cent	Number	Per cent
0–54	10	12 1	10	10 6
55–64	18	21 7	19	20 2
65–74	38	45 8	40	42 6
75+	17	20 4	25	26 6
All ages	83	100	94	100

(b) *Social class*—Table VII shows that the distribution of the cancer cases among the social classes follows that of the group of husbands of which they form part and is generally similar in both groups   Nevertheless, a relatively

TABLE VII —*Social Class Distribution of Cancer Cases in the Two Groups of Husbands*

	SOCIAL CLASS					Not stated	Total
	1	2	3	4	5		
Cancer cases among Husbands of Cancer Widows							
No	4	12	42	11	12	2	83
%	4 8	14 6	50 4	13 3	14 5	2 4	100
Cancer cases among Husbands of Non cancer Widows							
No	5	9	48	19	12	1	94
%	5 3	9 6	51 0	20 3	12 7	1 1	100

larger number of cases fall into Group 2 and a small number into Group 4 amongst the pairs where both husband and wife died from cancer than amongst the pairs where the husband only died from cancer

(c) *Geographical distribution of cases of cancer in the husbands*—Table VIII shows the distribution amongst the 6 areas in the survey of the 83 cases of cancer in the husbands of cancer widows and the percentage in each area It also shows the distribution amongst the same areas of the 94 cases of cancer in the husbands of non-cancer widows Comparison of the two groups shows that the percentages were very similar in all areas except Gosport where the numbers were very small It is also seen that in Banstead the percentage of cancer in both groups, i e 31 5 per cent and 33 3 per cent, was higher than the total percentages (20 per cent and 20 6 per cent respectively)

TABLE VIII —*Numbers and Percentages of Husbands in Each Area who Died From Cancer, for Each of the Two Groups of Widows*

	Cancer widows			Non cancer widows		
Districts	Total	With cancer husbands	Per cent	Total	With cancer husbands	Per cent
Gosport	9	1	11 0	13	4	30 8
Eastleigh	56	14	25 0	63	11	17 5
Winchester	34	5	14 7	32	5	15 6
Banstead	38	12	31 5	27	9	33 3
Merton and Morden	94	21	22 3	109	26	23 8
Fulham	186	30	16 1	211	39	18 5
All districts	417	83	20 0	455	94	20 6

Association of Specific Sites of Neoplasm in Husband and their Wives

The above remarks apply to cancer of all sites taken together   An examination was also made to see whether the husbands of women dying from growths of particular sites were themselves more likely than might be expected by chance (i e more than the husbands of wives who did not die from cancer would be) to die from the same particular site growth, or cancer of some other site   An account of this is given in the Appendix and Table IX

DISCUSSION AND COMMENTS ON RESULTS

It will be seen from the above results that the percentage of husbands of cancer widows who also died of cancer did not differ from the percentage of husbands of non-cancer widows who died from cancer   It appears then that the occurrence of cancer in the wives was not linked with the occurrence of cancer in their husbands who pre-deceased them   To put matters simply, if a wife died from cancer there was no more chance that her husband would die from cancer than if he were the husband of someone else who did not die from cancer   Therefore, there is no evidence, from this investigation (at this time and place), that habits common to husband and wife have detectable importance in causation of cancers from which both could suffer

TABLE IX

Diagnosis	All cancer widows	Husband and wife with same growth		Non cancer widows' husbands who died from growth of these sites	
		No	%	No	% (of 455)
Tongue	1	—			
Floor of mouth	1	—			
Other parts of mouth	1	—			
Hypopharynx	1	—			
Oesophagus	6	—			
Stomach	65	3	4 6	17	3 7
Small intestine	2	—			
Large intestine exc rectum	49	1	2 0	13	2 9
Rectum	36	1	2 8	10	2 2
Extra hepatic ducts	14	—			
Liver (secondary)	3	—			
Pancreas	13	—			
Peritoneum	5	—			
Larynx	2	—			
Trachea, bronchus and lung	19	2	10 0	20	4 3
Mediastinum	2	—			
Breast	77	—			
Cervix uteri	20	—			
Corpus uteri	5	—			
Uterus, unspecified	14	—			
Ovary and fallopian tube	21	—			
Unspecified fem gen organs	2	—			
Kidney	4	—			
Bladder and ur m. organs	11	—			
Malignant melanoma of skin	1	—			
Other malignant neoplasm of skin	3	—			
Eye	1	—			
Brain and other parts of N S	4	—			
Thyroid gland	2	—			
Bone (including jaw bone)	3	—			
Secondary and unspec malig neoplasm of lymph nodes	1	—			
Other and unspecified sites	16	—			
Lymphosarcoma and reticulosarcoma	2	—			
Hodgkin's disease	3	—			
Lymphatic leukaemia	1	—			
Myeloid leukaemia	2	—			
Acute leukaemia, type unspecified	4	—			
	417	7			

N B —To reduce the complexity of presentation the detailed site distribution of all cancer deaths in the two groups of husbands is omitted from this Table   It is available on request

Thus conclusion is not invalidated by the fact that we cannot draw up a hard and fast list of habits that are or are not always either individual or common to husband and wife

The above remarks are likely to be true even if we assume that a complex of factors in heredity and environment (both inside and outside the home) is needed to set the stage necessary for a particular growth to occur

Can our negative result be interpreted as meaning that, in future endemiological investigations based on questionnaires, we can exclude inquiry into domestic habits usually common to members of the same household? It might, but because

this could lead to neglect of a large field of inquiry it would be safer to say " The negative result suggests that an investigation of domestic habits (in relation to cancer aetiology) would be more likely to be profitable if directed towards habits usually peculiar to individuals in a household than towards those common to most members of the household "   Nevertheless, although this wide, negative finding might guide future investigations, it should not limit their field, lest some important fact should, by neglect, fail to be revealed

<div align="center">CONCLUSION</div>

There is no evidence, from the present investigation, to confirm the hypothesis that domestic factors or habits common to husband and wife are carcinogenic
The results seem to suggest that it would be less profitable to investigate the possible carcinogenic influence of common domestic factors than other, usually unshared, factors   However, this conclusion should be applied with caution to future plans for endemiological enquiries lest a conceivably important field for further investigation be neglected

<div align="center">SUMMARY</div>

This investigation attempts to answer the question " How important are shared domestic environmental factors in the aetiology of cancers?" To eliminate family genetic factors a study was made of the causes of death of husbands and wives
The material consisted of 417 widows who died from cancer (" cancer widows ") and their husbands, the causes of all the deaths being known   A carefully matched control group of 455 widows who did not die from cancer (non-cancer widows) together with their husbands, the causes of whose deaths were also known, was used for comparison   The cancer widows and control widows were closely comparable in respect of age, social class, place of residence, date of death, etc
It was found that 20 per cent of the husbands of cancer widows died from cancer   The percentage of husbands of non-cancer widows who died from cancer was 20 6 per cent
It is concluded that, as cancer in the husbands of women who died from cancer was no more frequent than cancer in the husbands of women who did not die from cancer, then it is unlikely that shared domestic environmental factors are important carcinogenic agents for the time, places and people in this study
An appendix describes the observed percentage of cancers of identical sites in cancer widows and their husbands and compares these with the findings in the group of non-cancer-widows and their husbands

NOTE

When this paper was written the literature was searched under the heading " Cancer in Husbands and Wives " without result   Because the author happened to come across a reference to Ciocco's work the literature was re-searched under the heading " Mortality in Husbands and Wives "   This revealed Ciocco's (1940, 1941, 1942) papers   Using an approach similar but not identical to the method described in the present paper Ciocco found an excess of cancer above expectation in the spouses of cancer subjects, i e his results are the reverse of our own

Evelyn A Potter and Mildred R Tully, deliberately following Ciocco's methods as closely as they could in Massachusetts, failed to confirm his results Their findings, then, are compatible with our own, though they used a somewhat different method

Obviously from the above conflicting results further investigations are required and until these have been carried out, generalisations about the presence or absence of association of cancers in husbands and wives are unjustifiable

Thanks are due to the following Medical Officers of Health for allowing access to their records for this research

Dr E J MacIntyre   (Banstead U D C)
Dr W Alastair Glen   (Eastleigh M B)
Dr M I Adams   (Fulham M B)
Dr Percival W Pritchard (Gosport M B)
Dr W D Swinney   (Merton and Morden U D C)
Dr Robt A Good   (City of Winchester)

My special thanks are due to Miss Joyce Strudwick and Miss Rosemary Axe, B A who carried out most of the investigation   Thanks for co-operation are due to Miss V P Farrow and her staff at the South West London Mass X-ray Service, especially to Mrs V Collins, Mrs M Levett, Mrs W E Smith, Miss A Taylor, Mrs V Thomas and Miss K E Janvrin Tims

I am also grateful to the Librarian of the Royal Society of Medicine for searching the literature

I am required to state that this investigation formed part of a successful entry for the South West Metropolitan Regional Hospital Board Research Prize Competition 1958

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APPENDIX

Table IX shows the type occurrence of the 417 cancers occurring in the cancer widows (col 1)   Column 2 of this table shows the number of deaths from cancer of the same site occurring among the husbands of women dying from cancer of that particular site

Of 65 women who died from carcinoma of the stomach, 15 (23 per cent) had husbands who died from cancer of which 3 died from cancer of the stomach, i e 4 6 per cent, whereas of the 455 women not dying from cancer, 17 had husbands die from stomach cancer (3 7 per cent) and of the 352 women who died from cancer of other sites the husbands of 10 died from cancer of the stomach (2 8 per cent)

Of 49 women who died from carcinoma of the colon, 8 (16 4 per cent had husbands who died from cancer of which 1 died from cancer of the colon, i e 2 per cent whereas of the 455 women not dying from cancer 13 had husbands die from carcinoma of the colon (2 9 per cent) and of the 368 women who died from cancer of other sites the husbands of 7 died from cancer of the colon (1 9 per cent)

Of 36 women who died from carcinoma of the rectum, 8 (22 per cent) had husbands who died from cancer of which 1 died from cancer of the rectum, i e 2 8 per cent whereas of the 455 women not dying from cancer 10 had husbands die from cancer of the rectum (2 2 per cent), and of the 381 women who died from cancer of other sites the husbands of 7 died from cancer of the rectum (1 8 per cent)

Of 19 women who died from lung cancer 5 (26 3 per cent) had husbands also die from cancer of which 2 died from lung cancer, i e 10 per cent, whereas of 455 women who died from causes other than cancer, 20 had husbands die from lung cancer (4 3 per cent), and of the 398 women who died from cancer of other sites the husbands of 17 died from lung cancer (4 4 per cent)

We have already described the absence of unexpected association of the same cancer in husband and wife in respect of the following sites — stomach, colon, rectum and lung   Looking down the list of remaining sites no example is seen of the same growth occurring in husband and wife among the less common growths

Examination of the series for evidence of associated causes of death in husbands and wives other than malignancy was undertaken but will not be described here, the number of diseases being so large in proportion to the number of deaths available that no reliable conclusions are possible   For the same reason it was not possible to make a reliable investigation into the possibility of an association between the occurrence of a particular site of cancer in the wife and some other specific non-malignant disease in the husband

THE INCREASE IN LUNG CANCER MORTALITY IN CANADA

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In a previous paper (Phillips, 1954) it was shown that in Canada between 1931 and 1952 deaths from lung cancer had increased approximately six times among males and had more than doubled among females The experience in numerous other countries in lung cancer mortality has been reported (Clemmesen, Nielson and Jensen, 1953 , Dorn, 1953 , Stocks, 1952) and equal or greater increases shown Such studies are based, generally, upon official mortality statistics However, in a disease which, for its correct identification, requires medical skills and facilities that are only recently coming into widespread use, the assessment of increases in lung cancer mortality requires a careful study of the relative accuracy of death certificates and special consideration for the predominant pulmonary diseases of the period under review

Greenwood (1948) has stated that a change of fashion in death certification due to increasing knowledge may affect comparability and more recently Gilliam (1955) has written that, in lung cancer, although a large majority of informed opinion accepts the belief that the increase being experienced in many countries is real, considerable divergence of opinion remains with regard to its magnitude Herdan (1958) has suggested that, when examining striking changes in the incidence of non-epidemic disease one must be mindful of the possibility of a change in diagnosis In order to study such comments as they apply to mortality data in Canada a review has been made of deaths attributed to the major diseases of the respiratory system for the period 1931–1956 The sexes have been analyzed separately between the ages of 35 and 84 years All mortality rates have been standardized on the 1951 census population of Canada in order to permit direct comparisons The following causes of death with the International List Number were selected for study —

I Malignant neoplasms of trachea, bronchus and lung not specified
as secondary—

47 (a), (b), (c), (d)	4th Revision
47 (a), (b), (c), (d), (e), (f)	5th Revision
162, 163	6th Revision

II Tuberculosis of the respiratory system—

23	4th Revision
13	5th Revision
0001 to 0008	6th Revision

III Diseases of the respiratory system—

104 to 114	4th Revision
104 to 114	5th Revision
A 87 to 97 (Intermediate List)	6th Revision

III (a) Bronchopneumonia—

107	4th Revision
107	5th Revision
491	6th Revision

III (b) Lobar pneumonia—
  108   4th Revision
  108   5th Revision
  490   6th Revision

III (c) Pneumonia (unspecified)—
  109   4th Revision
  109   5th Revision
  492, 493   6th Revision

The standardized death rates are shown for males in Table I and Figure 1 and for females in Table II and Fig 2 From the tables it will be noted that lung cancer mortality in males has increased from 6 9 per 100,000 population in 1931 to 56 9 in 1956, an increase of 8 2 times, while the female rates have increased approximately 2 4 times On the other hand the tuberculosis rates have decreased from 80 8 per 100,000 to 19 2 for males and from 64 2 to 8 7 for females All other respiratory diseases have decreased for males from 138 2 per 100,000 to 83 4 and for females from 125 7 to 44 0 per 100,000

TABLE I —*Standardized Death Rates Per* 100,000 *Population for Selected Causes Among Males 35 to 84 Years* (1931–1956)

Years	Lung cancer	T B	All respiratory	Pneumonia Broncho	Pneumonia Lobar	Pneumonia Unspecified
1931	6 9	80 8	138 2	34 4	42 0	22 3
1932	7 4	75 5	138 0	36 9	45 7	22 6
1933	8 8	72 1	135 8	36 9	42 0	18 5
1934	8 6	72 2	135 0	35 7	45 0	15 0
1935	10 7	73 9	141 8	40 2	53 0	15 1
1936	10 9	72 9	144 6	44 3	52 8	14 7
1937	12 1	70 6	148 0	44 3	52 6	13 5
1938	12 4	65 1	140 6	40 2	54 0	13 0
1939	15 5	64 7	122 0	41 7	36 7	11 6
1940	17 3	64 4	114 5	36 7	32 9	9 9
1941	17 6	69 6	93 0	29 4	24 1	11 1
1942	18 9	66 1	90 4	30 3	25 2	10 7
1943	18 7	69 2	101 8	31 2	30 7	11 9
1944	18 5	63 4	91 8	28 6	26 2	11 7
1945	21 3	61 6	81 8	24 8	22 8	10 7
1946	24 4	61 2	84 8	27 5	19 6	10 7
1947	28 4	64 9	78 7	27 5	18 5	9 1
1948	29 5	57 6	78 5	27 7	18 8	9 0
1949	32 9	52 4	80 3	26 3	17 5	10 6
1950	36 9	46 4	73 3	20 2	12 2	8 0
1951	38 7	43 9	96 0	24 3	10 9	9 8
1952	43 0	33 4	68 4	22 0	8 1	7 1
1953	47 0	25 4	78 0	21 8	10 3	8 9
1954	50 1	24 0	66 4	20 7	8 3	8 2
1955	53 6	20 6	74 9	25 7	7 5	8 2
1956	56 9	19 2	83 4	27 2	10 3	7 9

The comments of Greenwood and Herdan regarding a change of fashion or a change of diagnosis in lung cancer death certification may be investigated by a study of the assumption that there has been no real increase in lung cancer mortality in Canada and that increases in death rates are due entirely to improvements in death certification If this be true then following the proposal of Gilham (1955), the number of deaths presumably resulting from cancer of the lung in 1931 may be calculated by applying the 1956 age-specific rates for each sex to
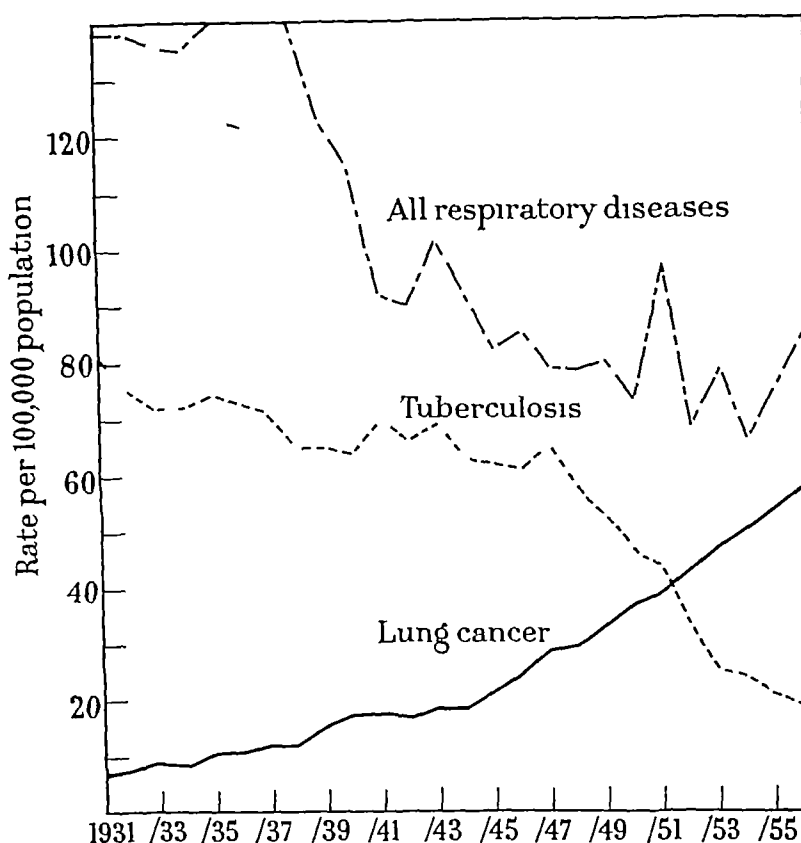
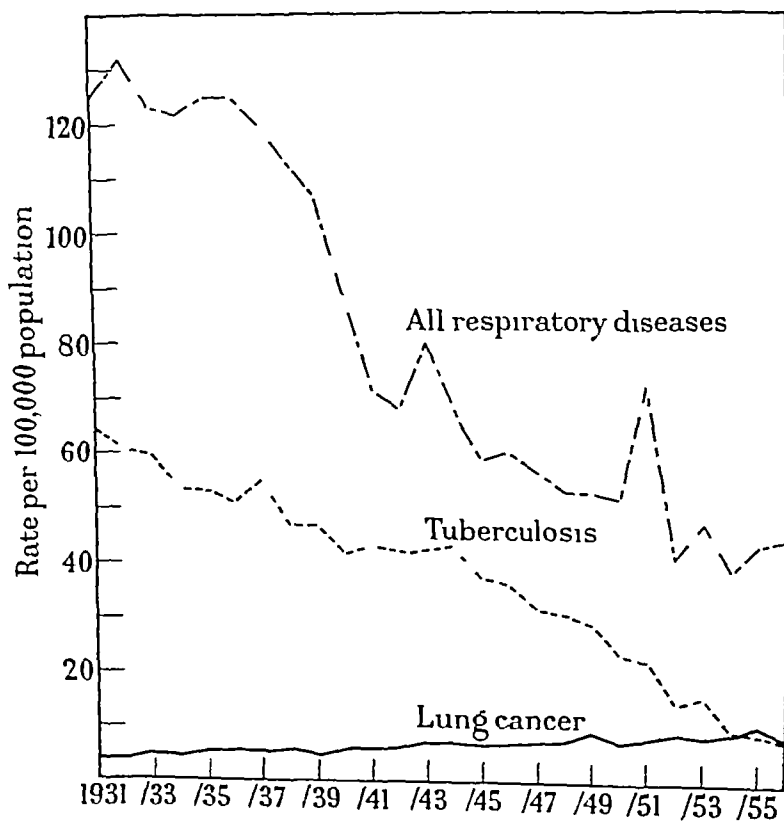Fig 1 —Standardized death rates for selected causes among males 35 to 84 years

Fig 2 —Standardized death rates for selected causes among females 35 to 84 years

TABLE II —*Standardized Death Rates Per* 100,000 *Population for Selected Causes Among Females* 35 *to* 84 *Years* (1931–1956)

Years	Lung cancer	T B	All respiratory	Pneumonia Broncho	Pneumonia Lobar	Pneumonia Unspecified
1931	3 8	64 2	125 7	38 4	33 6	22 1
1932	3 9	60 5	131 6	38 0	41 5	24 3
1933	5 0	59 6	123 8	39 9	35 5	18 2
1934	4 8	53 5	121 9	37 3	36 8	16 9
1935	5 4	52 9	124 8	41 8	41 1	15 0
1936	6 1	51 3	124 9	38 4	39 6	14 1
1937	5 6	54 6	120 0	42 5	41 6	12 0
1938	5 9	47 2	112 7	40 3	37 0	12 0
1939	5 2	46 7	107 3	38 6	31 8	11 1
1940	6 2	42 3	89 4	34 1	21 9	9 1
1941	6 2	43 2	71 6	23 3	18 5	10 1
1942	6 1	41 9	68 0	24 1	15 8	9 6
1943	7 4	42 5	79 7	27 6	19 6	10 6
1944	7 5	43 0	68 1	23 6	18 5	9 6
1945	7 0	37 4	58 9	21 0	14 2	7 7
1946	7 0	36 5	60 4	20 8	13 4	10 0
1947	7 4	31 8	57 0	22 5	12 0	7 8
1948	7 9	31 1	53 2	20 5	11 6	5 8
1949	9 5	29 3	52 8	20 0	10 0	8 3
1950	7 5	23 6	52 1	16 8	7 2	7 7
1951	8 2	22 5	72 4	19 2	5 7	7 0
1952	9 2	14 6	41 0	14 0	4 7	5 8
1953	8 5	11 1	47 5	16 5	4 8	5 2
1954	8 9	10 0	38 5	14 4	4 5	5 4
1955	10 8	9 8	43 0	16 2	4 3	5 4
1956	9 2	8 7	44 0	16 8	4 3	7 0

the appropriate 1931 populations   The results so obtained, less the deaths actually recorded in 1931 give the deaths from cancer of the lung that on this assumption were, in 1931, incorrectly attributed to some other disease   These are given for males and females in Table III

TABLE III —*Additional Deaths from Lung Cancer Needed in* 1931* *Shown as Percentages of Deaths from all Respiratory Diseases Including Tuberculosis*

Age Group	Additional lung cancer deaths needed in 1931 Male	Additional lung cancer deaths needed in 1931 Female	Additional lung cancer deaths as proportion of deaths from all respiratory diseases Male (%)	Additional lung cancer deaths as proportion of deaths from all respiratory diseases Female (%)
35–39	6	2	1 6	5 3
40–44	20	0	4 8	0 0
45–49	40	5	9 2	2 0
50–54	95	6	22 0	2 9
55–59	140	14	39 0	7 0
60–64	158	6	46 6	2 6
65–69	165	13	41 5	4 3
70–74	123	16	30 1	4 1
75–79	59	14	17 0	3 7
80–84	31	5	10 8	1 5

* In order that the 1931 and 1956 lung cancer mortality rates be equal

It would seem reasonable to assume that deaths from cancer of the lung might be confused more frequently with tuberculosis and other respiratory diseases, than with any other cause   If there had been no increase in lung cancer deaths

between 1931 and 1956 and the additional deaths needed in 1931, shown in Table III, had been erroneously diagnosed as some other respiratory disease including tuberculosis, then these needed deaths divided by the recorded deaths for respiratory diseases including tuberculosis give the percentages of error in diagnosis of these diseases necessary in each age and sex group These percentages are given in Table III and show that for the age-specific lung cancer rates to remain the same the diagnostic error in all respiratory diseases including tuberculosis would have ranged from 1 6 per cent, for males aged 35 to 39 years to 46 6 per cent for those aged 60 to 64 years In addition different percentages of error would have been required for females In order that the basic assumption be upheld viz that increases in lung cancer death rates are due entirely to improvements in death certification, it is necessary to show that errors in diagnosing lung cancer as other respiratory diseases occurred more frequently in males than females and more frequently in the older age groups Since there is not a consistent increase of diagnostic error with age the basic assumption must be rejected and the data interpreted to indicate that an increase in cancer of the lung has in fact occurred

It is difficult to determine precisely how well the recorded mortality measures the magnitude of this increase Numerous examples have been presented, especially in earlier years, showing the erroneous diagnosis of lung cancer as tuberculosis, and post-mortem studies such as reported by Waller and Grimstvedt (1958) indicate that a substantial diagnostic error may occur in carcinoma of the lung More recently the use of antibiotics has permitted a number of patients to survive until the correct diagnosis was made whereas they would have succumbed formerly to pneumonia or some other respiratory cause before the discovery of the underlying cancer of the lung Unfortunately there is no way to estimate directly to what extent such circumstances have affected the recorded increase in lung cancer This may be done indirectly by measuring the effect of a selected percentage of error in the diagnoses of respiratory diseases on the mortality trend of lung cancer Assuming a 2 per cent and a 5 per cent error of diagnosis the deaths resulting from the assumptions are deducted from the recorded number and transferred to cancer of the lung

Table IV shows the results of these calculations and it will be noted that, whereas in males the recorded lung cancer mortality rates increased 8 2 times, an error of 5 per cent reduces this to 3 6 times In females the increase is reduced from 2 4 times, to no increase If a 2 per cent error in diagnosis is assumed the increase in lung cancer mortality for males is 5 3 times and 1 4 times for females This procedure assumes that the error factor is constant throughout the period under review whereas it would seem more reasonable to assume that errors in

TABLE IV —*Effect of a Five Per cent Error in the Diagnosis of Respiratory Diseases on the Mortality From Lung Cancer*

Sex	Standardized lung cancer death rate (1931)	Standardized lung cancer death rate (1956)	Number of times increase	Standardized lung cancer death rate including 5% error (1931)	Standardized lung cancer death rate including 5% error (1956)	Number of times increase
Male	6 9	56 9	8 2	17 6	64 0	3 6
Female	3 8	9 2	2 4	13 4	11 9	None

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diagnosis have decreased in recent years If the 5 per cent error factor is applied only to 1931 deaths and the 1956 deaths assumed to contain no error the increase in lung cancer mortality in males would be reduced further, to 3 2 times

It is to be noted also that an assumption of a constant error in the diagnosis of all respiratory diseases including tuberculosis results in a decrease in the error in diagnosis of lung cancer Referring to Table IV the calculated lung cancer rate in 1931, assuming an error of 5 per cent in respiratory disease diagnoses, is 17 6 for males and 13 4 for females while the recorded rates are 6 9 and 3 8 Thus on this assumption 39 2 per cent (6 9/17·6) of the male lung cancer and 28 4 per cent (3 8/13 4) of the female were correctly diagnosed in 1931 In 1956, however, the corresponding proportions are 88 9 per cent (56 9/64 0) for males and 77 3 per cent (9 2/11 9) for females It seems evident therefore that, as deaths from all respiratory diseases decline there is a decreasing possibility that errors in diagnosing them could contribute substantially to the death rate from lung cancer

Whether the two per cent or the five per cent error in diagnosis is considered acceptable it seems reasonable to assume that errors in the diagnosis of lung cancer occur and may have occurred more frequently twenty-five years ago On this assumption the increases, since 1931, in the recorded lung cancer death rates in Canada for both sexes are probably higher than in actual fact

SUMMARY

A study has been made of the experience in Canada in deaths from lung cancer and other major respiratory diseases The period reviewed was 1931 to 1956 The standardized death rates indicate that lung cancer has increased approximately eight times among males and doubled among females Significant declines for each sex were found in deaths from tuberculosis and from all other respiratory diseases The assumption that all of the increase in mortality attributed to lung cancer since 1931 could be accounted for by erroneous death certification to other respiratory diseases including tuberculosis cannot be substantiated without unreasonable assumptions of age and sex differences in diagnostic error It is shown also that a substantial proportion of the recorded increase can be accounted for theoretically by a diagnostic error of five per cent Thus if five per cent of the deaths attributed to respiratory diseases including tuberculosis were actually due to lung cancer, the recorded increase in males since 1931 in Canada would be 3 6 times instead of 8 2 times and in females no increase would be evident instead of one of 2 4 times The increase would be reduced somewhat further on the assumption of greater diagnostic error in the earlier than in the later years under review

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MALIGNANT DISEASE OF THE GASTRO-INTESTINAL TRACT IN SINGAPORE

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SINGAPORE is singularly suitable, as is neighbouring Malaya, for the study of what has become known as Geographical Pathology The presence of a mixed population of some one and a half millions, 75 per cent Chinese, 14 per cent Malay, and 9 per cent Indian and Pakistani, within an island of some 217 square miles, makes for an epidemiological unit of convenient size, all the more so as there is but one general hospital Since the island lies one degree north of the equator, and one hundred and four degrees east of Greenwich, the climate is hot (average 85° F), moist (average relative humidity 83 per cent), and equable There are no seasons

METHODS MATERIAL SOURCES OF ERROR

At the mortuary of the General Hospital, Singapore, post-mortem examinations are carried out on persons who have died in the hospital, and on cases falling within the aegis of H M Coroner In addition, a morbid anatomist from this department visits the Kandang Kerbau Maternity Hospital post-mortem records are thus available from these three sources A few post-mortem examinations carried out at a tuberculosis and a military hospital are not included In short, records of almost all autopsies carried out in Singapore are available to this department

All the post-mortem records of cases of malignant disease were scrutinised by the author and the data considered relevant were entered on a specially prepared pro forma for subsequent analysis

This study covers the years 1948–58 inclusive, when 22,997 post-mortem examinations were carried out In this material there were 304 deaths from malignant disease of the gastro-intestinal tract, i e , 1 32 per cent If the 11,525 children under the age of 11 years be excluded then the incidence is 2 65 per cent

The age and sex incidence, by race, for the post-mortem population for the years 1950–58 inclusive is given in Table I, and the mean age and sex incidence, by race, for the entire population of Singapore for the period mid-1947 to mid-1957 is given in Table II As most values have been approximated to the nearest round number some of the cross-additions may be apparently incorrect

The sex disparity, once very great, in the population has been almost corrected since the cessation of unrestricted immigration However, it will be noted that there is still an undue preponderance of Indian males in the third and subsequent decades

Unfortunately, it has proved impossible to compile much of the data in this paper for the whole of the period covered by the post-mortem survey, and only such figures as were available have been presented

TABLE I —The Age and Sea Distribution of the Singapore Post-Mortem Population, 1950–58 Inclusive

Age	Chinese Male	Chinese Female	Malay Male	Malay Female	Indian Male	Indian Female	Others Male	Others Female	Total Male	Total Female	Grand total Number	Grand total Per cent
0–10	5,150	3,980	75	43	165	117	32	20	5,422	4,160	9,582	49 6
11–20	555	274	45	15	32	25	14	0	646	314	960	5 0
21–30	685	353	73	27	160	36	58	13	976	429	1,405	7 3
31–40	844	366	59	14	295	50	56	9	1,254	439	1,693	8 8
41–50	1,382	404	52	12	326	23	55	14	1,815	453	2,268	11 7
51–60	1,365	278	45	10	256	22	58	6	1,724	316	2,040	10 6
61–70	713	191	6	3	81	13	25	5	825	212	1,037	5 4
71+	101	95	10	7	15	4	9	5	225	111	336	1 7
Totals	10,885	5,041	365	131	1,330	290	307	72	12,887	6,434	19,321	100 1
	16,826 (87 0%)		496 (2 6%)		1,620 (8 4%)		379 (2 0%)		19,321			

TABLE II —Mean Age and Sea Distribution of the Singapore Population from Mid-1947 to Mid 1957

Age	Chinese Male	Chinese Female	Malaysians Male	Malaysians Female	Indians Male	Indians Female	Others Male	Others Female	Total Male	Total Female	Grand total Number	Grand total Per cent
0–9	143,040	133,308	25,478	25,707	11,187	11,004	3,718	3,031	184,323	173,730	358,061	30 0
10–19	97,085	80,150	14,140	14,754	7,268	5,305	2,020	2,234	121,410	111,500	232,927	10 5
20–29	90,194	66,460	18,222	14,262	15,518	5,420	2,982	2,702	105,917	88,855	194,772	16 3
30–39	50,800	54,540	12,477	5,063	17,903	3,226	3,074	2,502	93,317	69,113	162,431	13 6
40–49	53,054	45,015	7,103	2,580	10,034	1,502	2,100	1,561	73,250	53,202	120,453	10 6
50–59	32,102	28,270	3,725	1,254	4,597	703	1,103	935	41,077	32,480	74,100	6 2
60–69	12,388	15,074	1,436	739	1,101	272	435	453	15,360	17,054	32,414	2 7
70+	3,209	6,630	635		273	113	149	237	4,208	7,726	11,092	1 0
Totals	471,732	438,561	83,225	73,139	68,842	27,071	15,731	14,315	639,520	553,687	1,103,216	90 0
	910,293 (76 3%)		150,364 (13 1%)		96,513 (8 1%)		30,046 (2 5%)		1,103,216			

It will be seen that there is considerable disparity between the post-mortem rates for the various races, the relative racial proportions for the population as a whole being Chinese 76 3 per cent, Malay 13 1 per cent, Indians 8 1 per cent, and Others 2 5 per cent, while the corresponding figures for post-mortems are 87 0, 2 6, 8 4 and 2 0 per cent

Hence, unless the disease process under study is likely to cause sudden death, when an autopsy would be carried out irrespective of race at the mandate of H M Coroner, any post-mortem series will be quite unrepresentative racially, as for religious and other reasons Malays virtually never give consent for autopsy Thus great care must be taken to ensure that the figures are comparable before concluding that any given neoplasm or disease process is rare or common in a particular racial group Further, the requirement for autopsy in cases of sudden death in a racial group which is averse to morbid examination will give a false impression of the importance as a cause of death of, say, coronary heart disease

The age structure of the Singapore population tends to give a falsely low impression of the relative incidence of malignant disease, just over half the population being under 21 years of age (1957) Further, the rate of natural increase is such that by 1962 half the population may well be under 15 years

Over the years 1906–10, when the mean population was about 250,000 the cancer mortality rate per 100,000 population was 12 6 (Hoffman, 1916) For the period 1926–31, when the population was approximately double, the incidence was 40 6 (Hoffman, 1935) In 1957 the overall incidence was 53 2, and for the preceding five years the mean rate was 51 9 This gradual rise and maintenance of the rate of incidence at a time of explosive natural increase suggests either that the incidence of cancer as a whole is rising, or that the public is making better use of the improved medical facilities

As post-mortem figures of incidence, both absolute and relative, are usually held to be biased, it was felt that no fair comment could be made unless hospital admissions, hospital deaths and the returns of the Registrar-General, Singapore, gave roughly comparable figures That they do so tally to a remarkable degree

TABLE III —*Comparison of the Number and Relative Incidence of Hospital Admissions, Hospital Deaths, Post-mortems, and Deaths recorded by the Registrar-General, Singapore, for Malignant Tumours of the Gastro-Intestinal Tract, 1954–58 Inclusive*

International List No		Hospital admissions		Hospital deaths		Post mortems		Registrar-General	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
150	Oesophagus	485	27	172	34	48	32	299	24
151	Stomach	856	48	253	50	87	58	712	58
152	Small gut	12	1	1	—	2	1	2	—
153	Large gut	179	10	43	8	7	5	112	9
154	Rectum	241	14	39	8	6	4	109	9

is shown in Table III As the system of classification of the returns of both the hospital and the Registrar-General has altered several times in the past ten years it was not possible to complete this table for the period covered by the post-mortem survey

The figures of the Registrar-General, Singapore, are probably the most accurate in South-East Asia as 55 per cent of deaths are certified by doctors, 12 5 per cent by H M Coroner, usually following a post-mortem examination, and 32 5 per cent by Police Officers and Hospital Assistants (1957)   In neighbouring Malaya, in 1956, 80 7 per cent of deaths were not certified by a medical practitioner   The majority of deaths certified by unqualified persons are ascribed to " sawan " (convulsions) or " demum " (fever), (Griffith, 1958)   Neoplasia is rarely, if ever, mentioned

Neoplasms of the gastro-intestinal tract form a high proportion of all malignant tumours   In Table IV the incidence of these tumours, as recorded by the Registrar-General, Singapore, is given, and expressed as a percentage of the 3,777 malignant neoplasms recorded in the same period

TABLE IV —*Incidence of Gastro-Intestinal Tract Neoplasms Recorded by the Registrar-General, Singapore, 1952–57 Inclusive*

Site	Number	Per cent	Percentage of all malignant neoplasms, i.e of 3777
Oesophagus	283	22 0	7 5
Stomach	783	61 0	20 7
Small intestine	6	0 5	0 2
Colon	109	8 5	2 9
Rectum	103	8 0	2 7
Total	1284	100 0	34 0

The oesophagus

There were 84 cases of oesophageal carcinoma, 73 males (87 per cent) and 11 females   The mean age at death for the 11 females, who were all Chinese, was 56 1 $\pm$ 13 4 years, and for the 69 Chinese males 53 9 $\pm$ 9 4 years, 2 Indian Hindus, 1 Indian Muslim and 1 Malay brought the male total to 73

The peak incidence for males was in the decades 40–49 and 50–59, while for the females it was in the decades 50–59 and 60 –69

Ochsner and de Bakey (1941) who collected 8572 cases of oesophageal carcinoma from the medical literature found that 20 per cent were situated in the upper-third, 37 per cent in the middle third, and 43 per cent in the lower third   Comparable figures for this series are 6 per cent, 50 per cent and 40 per cent   The site was not stated in the remaining 4 per cent

The majority of the carcinomata were described as being about 10 cm in diameter   obstruction was noted in 72 per cent

Fistulae were common, occurring in 40 per cent of upper third, 38 per cent of middle third, and 10 per cent of lower third carcinomata   Those in the upper third communicated with the trachea , those in the middle third with the lung direct, the bronchi, the trachea and the pericardium in that order , those of the lower third with the lung direct and the pericardium

Not unexpectedly, abscess formation was common   Fifteen per cent of un-operated cases showed a concomitant lung abscess, 5 per cent a mediastinal abscess and 2 per cent a brain abscess

Metastatic spread was extremely common The mediastinal glands were grossly involved in 45 per cent of cases, the abdominal glands in 18 per cent and the cervical glands in 11 per cent There was spread to local tissues in 30 per cent, to the lung in 13 per cent, to the liver and brain in 10 per cent of cases These figures are in rough agreement with those of Dormanns (1939) who analysed the sites of metastasis in 824 cases of oesophageal carcinoma

Oesophageal carcinomata seem to be much commoner in Singapore than in the Occident, forming 27 6 per cent of this series, and about 8 per cent of all neoplastic deaths (Table IV)

Ackerman and Regato (1954) state that in the U S A approximately 2 per cent of all deaths from carcinoma are oesophageal

Oesophageal growths have long been known to be very common in China Kwan (1937) found about half of gastro-intestinal growths were in the oesophagus, and a similar high incidence in the Chinese of Java has been noted (Kouwenaar, 1950)

Marsden (1958), in an analysis of biopsy material in Malaya, found carcinoma of the oesophagus in 19 per cent, 25 per cent and 17 per cent of gastro-intestinal tumours in Malays, Chinese, and Indians respectively

Much stress has been laid on the consumption of hot food as an aetiological factor and, as the Chinese male was reputed to be the first in the family to eat from the dish, the male preponderance was held to be accounted for This, as Marsden (1958) comments, is certainly not true for either Malaya or Singapore, and he suggests that the drinking of spirits is a much more likely cause The relatively high incidence of oesophageal cancer in Japan has also been attributed to alcohol (Irisawa, 1933) and Steiner (1956) discusses this relationship at length Despite the frequency with which the Chinese female in Singapore attempts suicide by drinking caustic soda, no history of such an episode was recorded in the 11 females, although in other reports the association has been noted (Bigger and Vinson, 1950)

The stomach

There were 164 cases of carcinoma of the stomach Of these 143 (87 per cent) were male, and 21 (13 per cent) female

One hundred and forty-seven were of Chinese race Of these 128 were male, and 19 female The mean age at death for the males was 51 7 $\pm$ 10 5 years and for the females 45 9 $\pm$ 11 7 years

The peak incidence was found to be in the decades 40–49 and 50–59 for the male (in all 67 per cent), and the decades 30–39 and 50–59 for the female (respectively 37 per cent and 32 per cent)

There were 13 cases in Indians, of whom 12 were male The mean age at death for the males was 48 2 $\pm$ 11 5 years, the peak incidence being in the decade 50–59

The sites of the tumours are given in Fig 1 It will be noted that there is some sex disparity in the incidence of greater curve tumours As the number of females is small the significance of this finding is doubtful Nevertheless an interesting point is raised which may repay further study

Evans (1956) finds that approximately 65 per cent of carcinomata are in the prepyloric region, 20 per cent on the lesser curve, and 4 per cent on the greater,

the remaining 11 per cent being found at either cardia or fundus   Oppolzer (1938) summarising 837 cases of gastric carcinoma found 54 per cent to be prepyloric, 29 per cent on the lesser curve, 7 per cent at the cardia, 2 per cent on the greater curve and 7 per cent to involve the entire stomach   The Singapore figures are thus substantially similar

Gross metastasis was very common, being present in almost every case   The commoner sites were the regional nodes in 54 per cent , the liver, by blood spread, in 33 per cent and, by direct extension, in 10 per cent , the omentum in 20 per cent , the para-aortic nodes in 11 per cent , the pancreas, lung, bone and brain each in 5 per cent   High as these figures are, they are considerably lower than
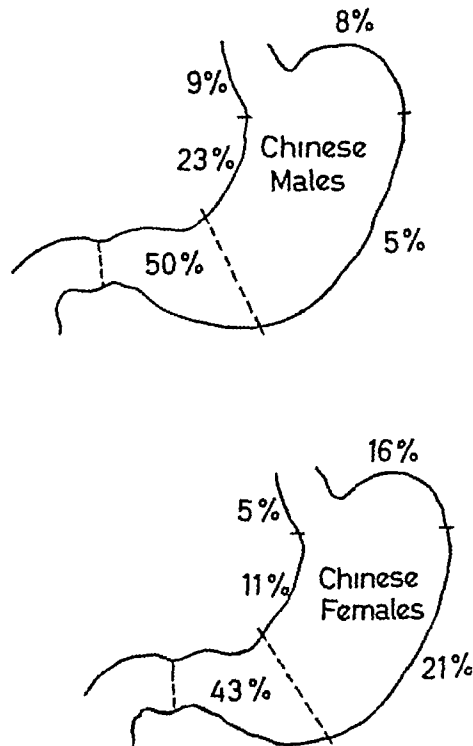
FIG 1 —The site incidence of carcinoma of the stomach in Chinese males and females

those of Stout (1943)   Ovarian metastases were seen in 37 per cent of the females Indeed, in one pregnant Chinese woman aged 40 years, the left ovary measured 28 by 16 by 10 cm , and weighed 2250 g , the right being approximately half this weight   Perforation of the malignant ulcer was observed in 25 cases (15 per cent), ascites in but 11 (7 per cent)

The returns of the Registrar-General, Singapore, show carcinoma of the stomach to be the commonest malignant tumour, accounting for one fifth of all deaths from malignant neoplasm   Marsden (1958) states that in Malaya the true incidence must be about 15 per cent   These figures are similar to those for the U S A where, in 1948, 12 6 per cent of all cancer deaths were due to a gastric carcinoma

Great stress has been laid on the frequency of gastric carcinoma in the Chinese as compared with the Malay, particularly in Java and Sumatra   In this series there were but two Malays (1 2 per cent) with gastric carcinomata   However, in Marsden's (1958) material the respective relative incidence for Malay, Chinese,

and Indians was 32 per cent, 35 per cent and 40 per cent of gastro-intestinal tumours, although on a population basis the relative incidence would be 5 per cent, 42 per cent and 53 per cent   It seems likely, in view of the strong aversion of the Malay to surgery, that the true relative racial incidence may in fact be much closer

However, in a series of 1301 post-mortems on Chinese males, and 1189 on Javanese males, Kouwenaar (1951) found 120 and 59 malignant tumours respectively   Forty-six (38 per cent) of the tumours in Chinese were in the gut (oesophagus 24 per cent, stomach 67 per cent, colon 7 per cent, rectum 2 per cent), whereas there were only four in the Javanese   These figures, and others quoted by Kouwenaar (1955), rather suggest that there is a true racial difference in the incidence of these tumours   It will be noted that the figures for the Indonesian and the Singapore Chinese correspond closely

In this series, if the incidence for male Chinese and male Indians + Pakistanis be compared with the mean male population for these races over the 11 years, then the relative incidence is virtually the same

Small gut

There were eight tumours of the small gut representing 2 6 per cent of the intestinal malignancies   Four of these all in males, were lymphosarcomata , the fifth in a female aged 38, was a sarcoma of doubtful origin , the sixth, in a male aged 30 years was due to Hodgkin's disease , the remaining two were adenocarcinomata occurring in a male aged 48 and a female aged 39 years   All were in persons of Chinese race

Colon

In this series there were 35 colonic cancers 13 males (37 per cent) and 22 females (63 per cent)   Of these, all but two were in Chinese

The average age at death of the males was 52 3 ± 12 8 years and of the females, 56 9 ± 9 3 years (A girl aged 9 years is excluded from this figure)

The peak decade of incidence was, 50–59 for males and 60–69 for females

Although the numbers involved are small there appears to be a sex difference in the site of the tumours as well This is shown pictorially in Fig 2 The sites are different to those found by Thompson (1957) in London

Metastases were common Fifty per cent of cases showed involvement of the regional nodes, 17 per cent extensive spread to local tissues, 11 per cent to the liver, and 6 per cent to the lungs Carcinomatosis was seen in 6 per cent Perforation was present in 46 per cent of cases and a major degree of intestinal obstruction in a similar proportion

The rarity of carcinoma of the colon in South East Asia has been commented on by Moore (1953) who spent five weeks in the region gathering the impressions of clinicians and pathologists

Although in other reported series (Bacon, 1945) the sex incidence has been M/F 1 2 1, in Singapore the ratio is 0 6 1 This disparity is all the more striking if it be noted that for the other gastro-intestinal tract tumours the ratio is 6 1. Marsden (1958) found carcinoma of the colon to account for 15 per cent of gastro-intestinal carcinomata He noted that there appeared to be no racial difference in incidence and that the M/F ratio was 1 5 1

Rectum

In this series there were 13 rectal cancers, 9 in males (69 per cent) and 4 in females (31 per cent)

For the eight Chinese males the average age at death was 53 4 ± 13 2 years and for the four Chinese females 54 3 ± 11 2 years

Metastases were common to local tissues 46 per cent, to regional lymph nodes 62 per cent, to the liver 31 per cent and to the lung 23 per cent Occlusion of the rectum was noted in 30 per cent Most tumours were stated to be about 5 inches (12 7 cm) from the anal margin
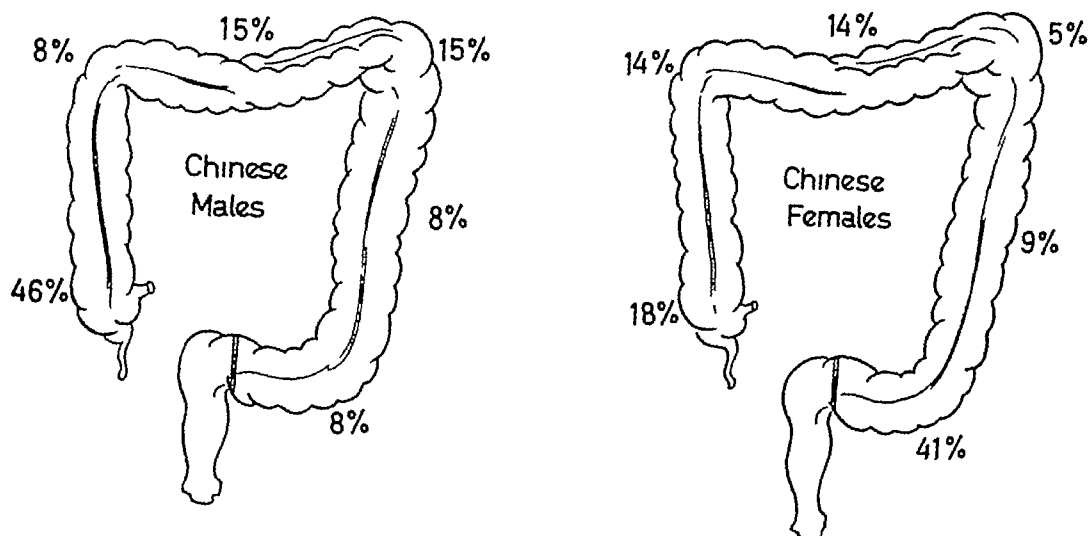
FIG 2 —The site incidence of carcinoma of the colon in Chinese males and females

DISCUSSION

It is of some interest to compare the post-war incidence of these tumours with pre-war figures

Although most of the records of the Department of Pathology were destroyed during World War II, it has proved possible to trace a few charts and papers relating to a paper read at a scientific meeting by Dr C Subrahmanyam, lately Senior Pathologist, Singapore

In 1937, 1939, 1940 and 1941, 383 post-mortems on persons having died with malignant disease were performed at Tan Tock Seng Hospital (This institution was at that time a teaching general hospital, but during the period covered by this paper has been a tuberculosis sanitorium) Of these, 145 (38 per cent) were gastro-intestinal, and of this 145, 43 per cent were oesophageal, 51 per cent gastric, and 6 per cent were found in the rectum and colon

The late Dr J C Tull, sometime Senior Pathologist, Singapore, provided comparative data for a paper by Bonne (1937) on the relative incidence of malignancy in the then Dutch East Indies In a total of 128 cancers, 50 were gastro-intestinal Of this 50, 30 per cent were oesophageal, 58 per cent were gastric, and 12 per cent were described as rectal

It would thus appear that whatever may be causing the increased absolute

incidence in cancer, the relative proportions of the various gastro-intestinal tumours have remained, by and large, much the same

In the U S A Boles (1958) has noted a gradual decline in the incidence, both absolute and relative, of gastric carcinoma, with an increased incidence of tumours of the colon

A comparison of the Singapore figures for gastro-intestinal neoplasms with those for England and Wales and the U S A is given in Table V The increased Singapore incidence of oesophageal, and to a lesser extent gastric neoplasms, and the diminished proportion of those in the colon and the rectum seems much too large to be fortuitous

TABLE V —*Comparison of the Relative Percentage Incidence of Gastro-Intestinal Tract Malignant Neoplasms, based on the Figures of the Registrar-General, Singapore, for 1952–58 inclusive, the Figures of the Registrar-General, England & Wales, for 1940–42 (Kennaway, 1950) and those of Boles, (1958) for the U S A in 1955*

Site	Singapore	E and W	U S A
Oesophagus	23 2	7 2	7 0
Stomach	59 5	41 9	35 0
Small gut	0 4	0 6	} 40 4
Large gut	8 7	30 9	
Rectum	8 2	19 3	17 5
Percentage all malignant neoplasms	33 8	47 5	40 0

Marsden (1958) eliminated the effect of the age structure of the Malayan population, which is very like that of Singapore, by plotting against the various age groups an index number derived from the ratio of cancer in each age group to the population in that age group each expressed as a percentage Fig 3 shows the age distribution of gastro-intestinal tract malignancies, corrected for population structure by this device The graph follows Occidental figures fairly closely, although the small number of old people in the population has some effect on the width of the curve, the maximum incidence thus being in middle age

Tempting though it may be to compare the Singapore figures with those reported from neighbouring territories in South-East Asia, such comparison is at best extremely crude Cooray (1954) has pointed out the difficulties in coming to a reliable estimate of cancer deaths in Ceylon, even on the basis of post-mortem figures, as only 6 per cent of hospital deaths come to post-mortem The figures of the Registrar (Ceylon) are also open to criticism as many deaths are certified by sanitary inspectors (Padley, not yet published) Biopsy figures for Ceylon are much more complete, but show what seems an unduly high proportion of rectal carcinomata, perhaps because such tumours are much more readily reached with the biopsy forceps Three per cent of 1815 malignant biopsies were gastro-intestinal , oesophagus 18 per cent, stomach 13 per cent, small intestine 4 per cent, colon 24 per cent, and rectum 40 per cent In South Vietnam a similar ' cancerogramme '' obtains (Joyeux and Nguyen-Van-Nguyen, 1953 , Nguyen-Van-Ai, 1958) Of 3118 cancers 2 5 per cent took origin in the gut , oesophagus 2 per cent, stomach 24 per cent, small intestine 13 per cent, colon 16 per cent and rectum 45 per cent While such figures are a necessary pre- liminary in the investigation of the malignant epidemiology of a region, they may well differ considerably from the true incidence Subrahmanyam (1953) records

Singapore biopsy figures for 1950 and 1951   In 595 malignant biopsies there were 54 from the gut , oesophagus 20 per cent, stomach 28 per cent, colon 28 per cent, and rectum 24 per cent   These values are at complete variance with the hospital, autopsy and Registrar-General's given in Table III
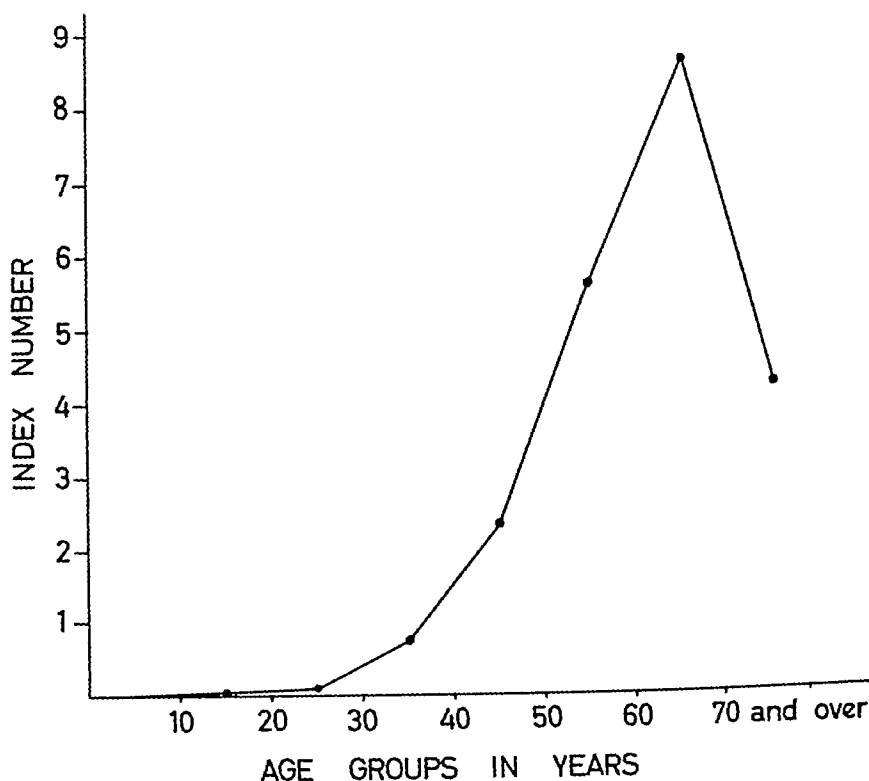

FIG 3 —The age distribution of gastro intestinal malignant neoplasms corrected for population structure

$$\text{Index number} = \frac{\text{Per cent cancer in each age group}}{\text{Per cent population in that age group}}$$

SUMMARY

The morbid features of a post-mortem series of 304 gastro-intestinal malignant neoplasms seen in Singapore over the years 1948–58 inclusive are described

The post-mortem incidence of these tumours is found to be   oesophagus 27 6 per cent , stomach 53 9 per cent , small intestine 2 6 per cent, colon 11 5 per cent , rectum 4 3 per cent   Comparison with hospital admission and death rates and with the figures of the Registrar-General, Singapore, for the same tumours, reveals a remarkable similarity

Comparison of the figures of the Registrar-General, Singapore, with similar data from England and Wales, and the U S A shows an increased frequency of oesophageal malignancies and a diminution of incidence of colonic and rectal tumours

Attention is drawn to the population and racial structure of Singapore and some of the fallacies attendant upon comparison of incidence of disease between racial groups are noted

The incidence of all malignancies has risen from 12 6 in 1906–10 to 51 9 per

100,000 population in 1952–6   No opinion can be given as to whether this rise is real or apparent

Over the years it appears that the relative incidence of the various gastro-intestinal tract tumours in post-mortem material has remained fairly constant

I wish to thank Professor R Kirk for kind help and encouragement, my colleagues in the University and Government Departments of Pathology for access to their post-mortem notes, Mr E J Phillips, Registrar-General, Singapore, for certain data, and Mr Daniel Liu for assistance with the calculations

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THE RELATIONSHIP BETWEEN AORTIC ATHEROSCLEROSIS AND CANCER

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A REDUCED degree and frequency of aortic atherosclerosis have been reported in patients with cancer compared with non-cancer controls (Foldes, 1949 , Wanscher, Clemmesen and Nielsen, 1951 , Juhl, 1955 , Creed, Baird and Fisher, 1955 , Elkeles, 1956) These observations have been made both on autopsied groups (Wanscher, Clemmesen and Nielsen, 1951 , Juhl, 1955, Creed, Baird and Fisher, 1955) and on groups of living patients (Foldes, 1949 , Elkeles, 1956) However, since death from cancer frequently follows prolonged malnutrition and starvation, conditions favoring remission of atherosclerotic lesions, conclusions relative to this hypothesis are open to question when based on autopsy observations On the other hand, Elkeles (1956) used roentgenologic diagnoses of calcification of the abdominal aorta to study the hypothesis in living patients This seemed reasonable since it has been shown that there is a high correlation between roentgenological and pathological findings (Hyman and Epstein, 1954) Elkeles confirmed the hypothesis that there is a dissociation between cancer and aortic atherosclerosis in general and extended it to include the concept that this dissociation holds primarily for cancers which are influenced by genetic elements or are hormone dependent but not for those caused by exogenous carcinogenic agents Unfortunately, the control groups utilized by Elkeles were drawn from general hospital populations and might well be selected with respect to the prevalence of aortic atherosclerosis

Since these observations may be of importance as an indication of the influence of various metabolic and hormonal factors on cancer and atherosclerosis, we thought it would be of interest to try to confirm them on a population of patients admitted to the Roswell Park Memorial Institute, a cancer hospital The advantage of this patient population is that, despite the fact that all patients admitted to the hospital have a suspect diagnosis of cancer, nearly half of the admissions are ultimately shown to be free of cancer This latter group of patients serves as a " control " for comparison with the cancer group

METHOD OF STUDY

As the study population, we selected 1462 consecutive admissions of white patients forty or more years of age during 1955 All patients had a posterior-anterior chest radiogram on admission , these were utilized for a diagnosis of aortic atherosclerosis X-ray films on 1405 (96 per cent) of the study group were located The radiologist (R L) read these films with respect to presence of athero-

sclerosis, without the knowledge of the age or diagnosis of the patient. Information on diagnoses and other characteristics of interest were subsequently abstracted and coded directly from the hospital charts. The frequency of atherosclerosis among the cancer patients was then compared with a similar frequency in the "control" group.

To check on the validity of the radiological diagnosis of aortic atherosclerosis, a separate study was carried out utilizing autopsy material as well as admission radiograms. The pathologist (J. P.) graded 192 aortas for severity of atherosclerosis. These determinations were repeated after an interval of two months without knowledge of the first determinations. The results of this dual reading of degree of aortic atherosclerosis are shown in Table I. In 72 per cent of the dual readings both determinations were the same. In only 3 instances did first and second determinations differ by more than one grade.

TABLE I —Comparison of First and Second Pathological Determination of Degree of Aortic Atherosclerosis; Atherosclerosis Severity Graded 0–4

First determination	Second determination					
	0	1	2	3	4	Total
0	—	—	—	1	1	2
1	—	17	12	—	—	29
2	—	1	29	18	—	48
3	—	1	5	63	5	74
4	—	—	—	10	29	39
Total	—	19	46	92	35	192

The radiologist (R. L.) then read the admission radiograms of these 192 patients without knowledge of the pathological diagnoses. The comparison of the radiological diagnosis of aortic calcification (atherosclerosis) and the first pathological diagnosis is shown in Table II. It is apparent that the radiological diagnosis was not particularly sensitive since a large number of patients with serious atherosclerosis were missed by the radiologist who depended on evidence of aortic calcification to make a diagnosis. However, the radiological diagnosis was highly specific since only cases with atherosclerosis of grade two or greater were diagnosed by the radiologist. In view of this specificity we considered that the radiological diagnosis of aortic atherosclerosis, based on a determination of the presence of aortic calcification, could serve as an adequate index to test the hypothesis of a dissociation between cancer and atherosclerosis of the thoracic aorta.

TABLE II —Comparison of Radiological Determination of Calcification of the Thoracic Aorta and First Pathological Diagnosis of Aortic Atherosclerosis

Radiological diagnosis	Graded pathological diagnosis									
	0		1		2		3		4	
	No	Per cent	No	Per cent	No	Per cent	No	Per cent	No	Per cent
Present	—	—	—	—	4	8.3	14	18.9	20	51.3
Absent	2	100.0	29	100.0	44	91.7	60	81.1	19	48.7
Total	2	100.0	29	100.0	48	100.0	74	100.0	39	100.0

RESULTS

Of the 1406 patients included in the study, 826 (59 per cent) ultimately were diagnosed as having cancer   Of the total sample 653 (46 per cent) were males and 753 (54 per cent), females   The age distributions of patients according to sex and presence or absence of cancer are given in Table III   Since there are differences between the age distributions of the males and females, both with and without cancer, it was necessary to take age into account in all comparisons between cancer patients and non-cancer patients , this was done by computing age-standardized percentages using the direct method of standardization

TABLE III —*Age Distribution According to Sex and Presence or Absence of Cancer Diagnosis*

Age	Cancer present		Cancer absent		Total	
	Number of individuals	Percentage distribution	Number of individuals	Percentage distribution	Number of individuals	Percentage distribution
Males						
40–49	46	11 0	49	20 8	95	14 5
50–59	110	26 4	65	27 5	175	26 8
60–69	131	31 4	68	28 8	199	30 5
70 and over	130	31 1	54	22 9	184	28 2
Total	417	99 9	236	99 9	653	100 0
Females						
40–49	94	23 0	123	35 8	217	28 8
50–59	96	23 5	99	28 8	195	25 9
60–69	112	27 4	71	20 6	183	24 3
70 and over	107	26 2	51	14 8	158	21 0
Total	409	100 1	344	100 0	753	100 0

In all previously published studies a difference between the overall frequencies of atherosclerosis among cancer patients and controls has been reported   Such a difference is not found in the present study   Table IV shows the age-specific proportions with radiological evidence of aortic atherosclerosis for both males and females with and without a diagnosis of cancer   For each age and sex these percentages are essentially the same for patients with cancer and for patients without cancer   The age-adjusted percentages for males are 18 5 among the cancer patients and 18 7 among the non-cancer patients, and for females they are 24 8 for cancer patients and 23 5 for non-cancer patients   It is interesting that the percentage atherosclerotic for each age group among both the cancer and non-cancer patients is greater for females than for males with the exception of the non-cancer group aged 50–59   This is in partial agreement with the observation of Elkeles (1957) that atherosclerosis of the abdominal aorta is more common among older women than among men of the same age

In Tables V and VI data for atherosclerosis of the thoracic aorta are presented according to sites and sex   Tables V and VI also indicate the difference between the age-adjusted percentages atherosclerotic for each of the site designations and

TABLE IV —*Radiological Evidence of Calcification of the Thoracic Aorta According to Age, Sex, and Presence or Absence of Cancer*

	Cancer present			Cancer absent		
	Number observed	With calcification		Number observed	With calcification	
Age		Number	Per cent		Number	Per cent
			Males			
40–49	46	2	4 3	49	—	—
50–59	110	11	10 0	65	8	12 3
60–69	131	27	20 6	68	15	22 1
70 and over	130	50	38 5	54	21	38 9
Total	417	90	21 6	236	44	18 6
Age adjusted*	—	—	18 5	—	—	18 7
			Females			
40–49	94	7	7 4	123	6	4 9
50–59	96	14	14 6	99	11	11 1
60–69	112	27	24 1	71	21	29 6
70 and over	107	56	52 3	51	24	47 1
Total	409	104	25 4	344	62	18 0
Age adjusted*	—	—	24 8	—	—	23 5

* Adjusted to age distribution of all males and females

for the non-cancer control group No striking differences between the age-adjusted percentages atherosclerotic of patients with cancer of various sites and controls are noted The largest difference (—10 per cent) is for white females with sites not specifically enumerated On the other hand, for cancers of the buccal cavity and pharynx among males the difference is positive that is cancers exceed controls by 9 per cent Of the 14 comparisons in both sexes, atherosclerosis is more frequent among cancer patients in 7 and less in 7

TABLE V —*Age-adjusted Percentages of Patients with Radiological Evidence of Calcification of Thoracic Aorta by Grouped Cancer Sites and Percent Difference Between Cancers and Controls, White Males*

Site	Int list numbers	Number observed	With calcification			Differences in age adjusted per cent, cancer patients minus controls
			Number	Per cent	Age-adjusted per cent	
Buccal cavity and pharynx	140–148	63	20	31 7	27 3	—8 6
Digestive organs and peritoneum	150–159	57	13	22 8	17 8	—0 9
Larynx, trachea, bronchus and lung	161–162	52	10	19 2	24 2	—5 5
Breast and genito urinary	170–180	36	9	25 0	22 7	—4 0
Skin	190–191	104	16	15 4	13 0	—5 7
Lymphatic and Hematopoietic	200–205	27	4	14 8	17 3	—1 4
All other sites	—	78	18	23 1	18 5	—0 2

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TABLE VI —*Age-adjusted Percentages of Cancer Patients with Radiological Evidence of Calcification of Aorta According to Site and Percent Difference Between Cancer Groups and Controls, White Females*

| Site | Int List numbers | Number observed | With calcification | | | Differences in age adjusted per cent, cancer patients minus controls |
			Number	Per cent	Age adjusted per cent	
Digestive organs and peritoneum	150–159	42	16	38 1	30 8	+7 3
Breast	170	117	35	29 9	30 3	+6 8
Cervix uteri	171	75	16	21 3	28 3	+4 8
Corpus uteri	172	27	8	29 6	27 3	+3 8
Other genito urinary	173–181	24	8	33 3	19 9	−3 6
Skin	190–191	30	8	26 7	20 7	−2 8
All other sites	—	94	13	13 8	13 7	−9 8

According to Elkeles (1956), aortic atherosclerosis is particularly infrequent in patients with gastric carcinoma (7 per cent), carcinoma of the breast (14 per cent), and prostate (10 per cent) On the other hand, he indicates that the occurrence of aortic atherosclerosis is the same in patients with cancer of the respiratory tract (38 per cent) and controls (37 per cent) In the Roswell Park series there were 15 patients with cancer of the stomach, and of these 3 (20 per cent) showed evidence of aortic atherosclerosis There were 117 patients with cancer of the breast, of whom 35 (30 per cent) showed evidence of atherosclerosis There were 16 patients with cancer of the prostate and 5 (31 per cent) of these showed aortic atherosclerosis Grouping cancer of the buccal cavity, pharynx, larynx, trachea, bronchus, and lung together yields 140 cases, of which 39 (28 per cent) had evidence of aortic atherosclerosis These data do not support the contention that particular cancer sites are relatively free of aortic atherosclerosis

In his studies of the dissociation between atherosclerosis and cancer, Elkeles (1956) used radiological evidence of calcification of the abdominal aorta as an index of atherosclerosis, since the abdominal aorta is usually the site of the earliest and most severe manifestations of aortic atherosclerosis Since various portions of the arterial system show different atherosclerotic manifestations, frequently uncorrelated, our observations of the thoracic aorta are not really comparable with observations of the abdominal aorta Of the 1462 patients in our sample, scout films of the abdomen were available on 509 (35 per cent) In addition, 115 abdominal radiograms were available for comparison with pathological diagnoses In Table VII the radiological diagnosis of abdominal aortic calcification is compared with the pathological diagnosis of abdominal atherosclerosis On the basis of routine scout films of the abdomen, the diagnosis of atherosclerosis of the abdominal aorta is shown to be somewhat less sensitive than for the thoracic aorta Only 11 per cent of patients with the severest grades i e grades three and four, aortic atherosclerosis were discovered by radiological examination of the abdomen Nevertheless, the technique was highly specific since none of the aortas graded zero or one by the pathologist was called atherosclerotic by the radiologist

In Table VIII the frequencies of atherosclerosis of the abdominal aorta for cancer patients are compared with the control group These data also failed to show any notable dissociation of aortic atherosclerosis between cancer patients

TABLE VII —*Comparison of Radiological Determination of Calcification of the Abdominal Aorta and First Pathological Diagnosis of Aortic Atherosclerosis*

	Graded pathological diagnosis									
	0		1		2		3		4	
Radiological diagnosis	No	Per cent	No	Per cent	No	Per cent	No	Per cent	No	Per cent
Present	—	—	—	—	1	3 2	2	5 3	5	19 2
Absent	2	100 0	18	100 0	30	96 8	36	94 7	21	80 8
Total	2	100 0	18	100 0	31	100 0	38	100 0	26	100 0

and non-cancer patients   For both sexes combined, the difference between the age-adjusted percentages with atherosclerosis is 7 per cent, with cancer patients showing more evidence of atherosclerosis than controls   Unfortunately, the number of patients was not sufficient for comparisons by each cancer site

TABLE VIII —*Radiological Evidence of Calcification of Abdominal Aorta According to Sex and Presence or Absence of Cancer, Total Percentages and Age-adjusted* Percentages*

	Cancer present				Cancer absent			
		With calcification				With calcification		
Sex	Number observed	Number	Per cent	Age adjusted per cent	Number observed	Number	Per cent	Age adjusted per cent
Male	162	31	19 1	17 4	78	7	9 0	9 0
Female	174	25	14 4	15 5	95	9	9 5	10 2
Total	336	56	16 7	16 4	173	16	9 2	9 6

* Adjusted to age distribution of all males and females

It is of interest to compare our findings and those of Elkeles (1956) with respect to calcification of the abdominal aorta among cancer patients   Since he published age-specific percentages, it has been possible to age-adjust his data and ours for comparison   When this was done, the frequency of aortic atherosclerosis among the cancer patients observed by Elkeles was the same as that observed at the Roswell Park Memorial Institute, namely, 16 per cent   We were also able to compare the percentages with atherosclerosis of the abdominal aorta in the control group in Elkeles' and the present series   Elkeles found atherosclerosis of the abdominal aorta among 35 per cent of his controls, while at the Roswell Park Memorial Institute 10 per cent showed atherosclerosis   These comparisons suggest that the differences between our findings and those of Elkeles may be due to differences in the types of patients used as controls   However, since Elkeles took special pains to diagnose calcification of the abdominal aorta, the observation of a similarity between the occurrence of this finding in his cancer series and ours may indicate an increased frequency of the condition in our series where no special effort was made to obtain radiograms for the diagnosis of abdominal aortic calcification

DISCUSSION

The hypothesis that atherosclerosis is less frequent among patients with cancer than among controls, and that this dissociation varies according to site, interested us since it was consistent with certain endocrine hypotheses for the etiology of selected cancers and was consistent with certain concepts regarding the etiology of atherosclerosis For example, Lilienfeld (1956) has shown that the excess of breast cancer in single women over married women may be attributed to the later entry into the menopausal state of single women and that this difference in age may be due to the increased frequency of artificial menopause in married women Winkelstein, Stenchever and Lilienfeld (1958) have shown that women with a history of myocardial infarction have a more frequent history of abortion and artificial menopause than a control group These two observations suggest the hypothesis that breast cancer and myocardial infarction are dissociated and would lead one to expect that breast cancer and atherosclerosis are likewise dissociated This is consistent with other data suggesting that estrogens have a protective effect with respect to coronary atherosclerosis and may serve as a predisposing influence on the development of breast cancer (Stamler, Katz, Pick and Rodbard, 1955 , Wuest, Dry and Edwards, 1953 , Rivin and Dimitroff, 1954) On the other hand, it has been shown that coronary atherosclerosis is not necessarily correlated with aortic atherosclerosis (Epstein, Boas and Simpson, 1957 , Pick, Stamler, Rodboard and Katz, 1952) so that failure to show a dissociation between aortic atherosclerosis and cancer of the breast does not shed light on the hypothesis that breast cancer and myocardial infarction are dissociated

The observations reported here which indicate that there is no dissociation between cancer in general, or cancer of particular sites, and atherosclerosis of the thoracic aorta are in sharp contrast to previously published data Nevertheless, the frequency of atherosclerosis of the abdominal aorta among the cancer patients surveyed in this study is similar to that observed by at least one previous investigator (Elkeles, 1956) It is our feeling that the differences observed in previous studies stem from two causes In studies of autopsied persons, cancer patients have been subjected to a variable period of starvation and malnutrition prior to death which may have produced a remission in the severity and frequency of atherosclerotic lesions Furthermore, control material drawn from autopsy studies would tend to be weighted by cases in which atherosclerosis might be a frequent accompaniment The second factor, i e, selection, may also be responsible for the differences observed in the radiological studies One would expect hospital populations to contain larger proportions of hypertensive and diabetic patients than the general population Epstein, Boas and Simpson (1957) have shown that diabetes mellitus is associated with an increased frequency of calcification of the aorta, determined radiologically, when compared with a control group without diabetes

It would seem that the utilization of patients suspected of cancer but subsequently shown to be without this disease as controls would eliminate some of the selective factors likely to be present in the study of a general hospital population It is unlikely that diseases such as diabetes and hypertension which are known to predispose patients to the development of aortic atherosclerosis would be selectively preferred among patients with suspected cancer, whereas they

would be expected to be more frequently represented in general hospital admissions than in the general population

It must be admitted that the difference in results between this study and that of Elkeles may reflect other factors whose frequencies differ in the United States and England However, we think that this is an unlikely explanation

Unfortunately, the present study does not adequately answer the question as to whether there exists a dissociation between the occurrence of coronary artery disease and cancer This more fundamental problem probably requires a prospective study A satisfactory answer could only be obtained by observing the occurrence of manifest coronary disease in a group of patients with various types of cancer and in a control group followed prospectively Comparison then of the frequencies of coronary disease in these groups would provide the answer with respect to the dissociation hypothesis

SUMMARY AND CONCLUSIONS

1 The frequency of atherosclerosis of the thoracic and abdominal aorta has been determined radiologically in a group of cancer patients and non-cancer patients

2 Posterior-anterior thoracic radiograms are highly specific in indicating the presence of calcification of the aorta while scout films of the abdomen are somewhat less sensitive but also highly specific

3 The frequency of calcification of the thoracic aorta and of the abdominal aorta has been shown to be essentially the same in cancer and non-cancer patients The percentages of cancer patients with radiological evidence of atherosclerosis of the aorta is essentially the same for various types and sites of cancer

4 The previously reported observation that there is a dissociation between atherosclerosis of the aorta in cancer patients and non-cancer controls is not confirmed in the present study

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A CASE OF PRIMARY OSTEOGENIC SARCOMA OF EXTRASKELETAL SOFT TISSUES

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METAPLASTIC bone formation in benign mesenchymal tumours is occasionally seen, as for example in fibromas and synoviomas (Evans, 1956, Willis, 1948), and in lipomas (Plaut, Salm and Truscott, 1959) Ossifying malignant neoplasms outside the skeleton have been recorded in many organs, but malignant osteogenic tumours arising primarily in the soft tissues are exceedingly rare Fine and Stout (1956), who reviewed this subject, found only one such case amongst their own material of over 156,000 specimens They were able to trace 34 primary osteogenic sarcomas of soft tissue in the literature, to which they added their own and a further 11 referred cases, making a total of 46 cases Yet in only 9 of these does the record include a post-mortem examination It is the purpose of this paper to present a complete record of such a case who was followed up closely since his first attendance

Case History

The patient, a farmer aged 51 years, presented himself in February 1958 with a large, raised, fungating mass above his left iliac crest He had been aware of it for the past 18 months and decided to seek medical advice only after seeing a television series eulogizing medical skill No evidence of metastatic spread was found in inguinal lymph glands and lungs Radiologically the growth appeared to contain bone, but was not connected with the skeleton The tumour was easily excised, it was embedded entirely in the soft tissues of the left loin and nowhere attached to or near to the iliac bone His wound healed well, but in July 1958 he had a haemoptysis and small secondaries were visualised for the first time on radiological examination of his lungs He soon started to lose weight, continued to have haemoptyses and his pulmonary secondaries steadily enlarged Left-sided inguinal glandular metastases developed from October 1958 onwards, ultimately attaining a large size with erosion of the overlying skin In January 1959 the serum alkaline phosphatase was found to be raised to 28 units His condition continued to deteriorate and he died of cachexia in August 1959, 18 months after excision of the primary growth and after a total illness of about 3 years

Operation specimen

The excised mass measured 15 × 9½ × 8 cm and was covered at one aspect by a spindle-shaped piece of skin This was bearing a broadly sessile, dark brown, patchily haemorrhagic tumour, measuring 6 cm in length and raised

4 cm above the skin surface   On slicing the growth was seen to extend up to
6 cm into the underlying fat tissue (Fig 1)   The excision had just cleared its
lower margin   Parts were necrotic   The better preserved areas were greyish
in colour and firm-elastic in consistency   Several small scattered bony areas
were noted as well as one large, excentrically placed, solid bony mass, measuring
5 × 3½ cm , which necessitated sawing

Necropsy

This was performed 10 hours after death   The operation scar was free from
recurrence   Large, necrotic glandular masses were present in the left groin,
covered by ulcerated skin and situated entirely within the inguinal soft tissues
Both lungs contained many secondary deposits (Fig 2), measuring from 2 to 10
cm across, which varied in consistency from soft to that of cartilage, and some
were almost solid bone   An occasional pulmonary metastasis had grown into
the bronchial lumen in polypoidal fashion causing partial obstruction   The
hilar lymph glands were not involved   The pancreas contained 3 small ossified
metastases measuring 1 cm in diameter, and a single larger, soft secondary,
measuring 3½ cm across   Five pedunculated or broadly sessile metastases were
found in the lower half of the duodenum and upper jejunum (Fig 3), measuring
from 1 to 5 cm across , two were hard and one of these had to be bisected with
the help of a saw   The brain contained a single, small, depressed metastasis
in the cortex of the right fronto-parietal region, and a further small pedunculated
deposit was found on the right parietal pleura   The skeleton was not involved ,
in particular a detailed examination of vertebral column, sacrum, wing of left
ileum and left pubic ramus failed to show any evidence of tumour growth

An alkaline phosphatase estimation, using heart blood, gave a reading of
140 units

Histology

Five blocks were cut from the operation specimen and 28 from the necropsy
material   The microscopical features of both primary growth and secondary
deposits were very similar   In the soft areas the tumour had the appearance
of a rapidly growing pleomorphic sarcoma with many tumour giant cells and
abnormal mitoses, growing either in diffuse sheets or displaying a fasciculated
arrangement (Fig 4)   In many sites the growing margins were sharply defined
(Fig 5), in others the neoplasm infiltrated diffusely into the surrounding sub-
cutaneous fat or adjacent alveoli   The presence of intrabronchial growth was
confirmed   the bronchial walls had been replaced, but the bronchial cartilage
and epithelium tended to be spared (Fig 6)   In the original growth as well as
in many secondaries of lung and in the pancreas the tumour showed much osteoid
tissue and bone, particularly dense bone having been formed by one of the pan-
creatic metastases (Fig 7 and 8)   Frequently the bony trabeculae were invested
with rows of malignant osteoblasts (Fig 9)   In addition small areas of malignant
cartilage were also in evidence and some of these were undergoing ossification
at their margins   Transformation of sarcomatous tissue into malignant cartilage
was noted in many areas   Where this was present adjacent to normal bronchial
cartilage the benign and malignant cartilaginous tissues were easily distinguishable
from each other

DISCUSSION

The present case appears to conform with many features brought out by Fine and Stout in their 1956 review   The average age of sufferers from extraskeletal osteogenic sarcoma of soft tissues is higher than that of osteosarcoma of bone and most patients are over 30 years of age   On the whole males appear to be affected more often than females and the extremities are involved more often than the trunk   Most tumours proved to be very malignant, the average 5-year cure being a little less than 9 per cent   Fine and Stout made the arbitrary distinction between osteolytic and osteogenic types, but emphasise that the quantity of bone present and the numbers of mitoses do not indicate the degree of malignancy nor the likelihood of metastases   In the present case anaplastic and osteogenic areas alternated with each other both in the primary growth and in the metastases   The lungs are the most common site of secondary spread, but in several instances widespread dissemination was recorded   Formation of malignant cartilage was noted in several instances

Osseous metaplasia is known to occur in reparative tissue, myositis ossificans, skin and other sites, and has been found in benign mesenchymal tumours, in the stroma of epithelial neoplasms and also in primary malignant mesenchymal growths   In all these tumours the tissues had no connection with bone and the sites precluded the presence of avulsed periosteum or osteoblasts   Bone formation must be regarded as due to divergent differentiation of the mesenchymal tumour cells themselves   This is in no way surprising   Whilst, as Willis (1948) has pointed out, the cell type of several kinds of mesenchymal tissue tends to be perpetuated in their tumours, when cells of mesenchymal origin multiply they can reacquire embryonic properties and redifferentiate in aberrant directions   Thus, in extraskeletal osteogenic sarcoma the proliferating tumour cells do not go on to produce, for example, collagen but differentiate into cartilage, osteoid and bone   This pluripotential property of the proliferating mesenchymal cell is most likely also the explanation of the so-called mesenchymoma, a term used to designate benign and malignant neoplasms made up of two or more types of mesenchymal tissue (Stout, 1948 ; Symmers and Nangle, 1957)

EXPLANATION OF PLATES

FIG 1 —Cross section through soft part of the primary growth showing haemorrhagic appearance and large " submerged " part   Small part projecting above skin surface About $\times \frac{1}{2}$

FIG 2 —Cut surface of left lung showing multiple secondary deposits   About $\times \frac{1}{3}$

FIG 3 —Two polypoidal jejunal metastases   Slightly reduced

FIG 4 —Primary growth   Anaplastic sarcomatous area with tumour giant cells   Haematoxylin and eosin. $\times$ 90

FIG 5 —Primary growth   Fairly sharp demarcation towards subcutaneous fat (bottom) Malignant cartilage at top left, osteoid formation at bottom right   Haematoxylin and eosin $\times$ 60

FIG 6 —Pulmonary metastasis   Bronchial cartilage enveloped by anaplastic sarcoma   The bronchial epithelial lining is still largely intact   Above   malignant cartilage and calcifying osteoid   Smaller islet of malignant cartilage below   Haematoxylin and eosin $\times$ 60

FIG 7 —Pancreatic metastasis showing dense bone formation   Malignant osteoblasts in lacunae   Haematoxylin and eosin $\times$ 60

FIG 8 —Pancreatic metastasis   Trabecular osteoid pattern   Haematoxylin and eosin. $\times$ 60

FIG 9 —Primary growth   Ossifying osteoid trabeculae with malignant cartilage at top Haematoxylin and eosin $\times$ 60
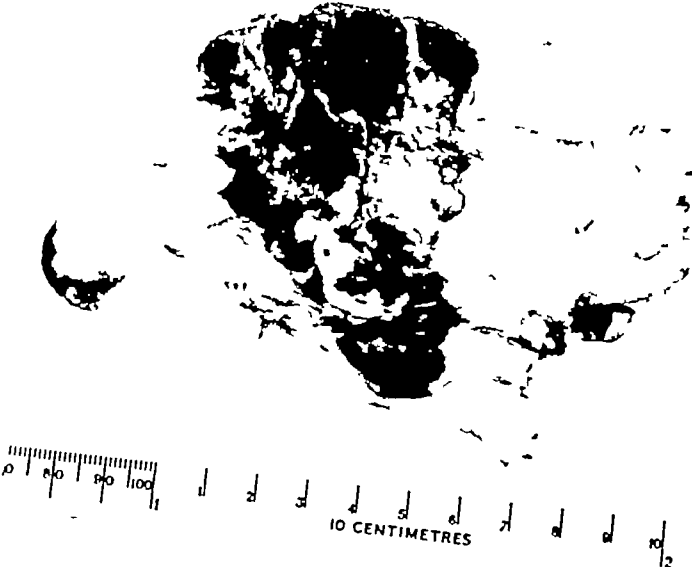
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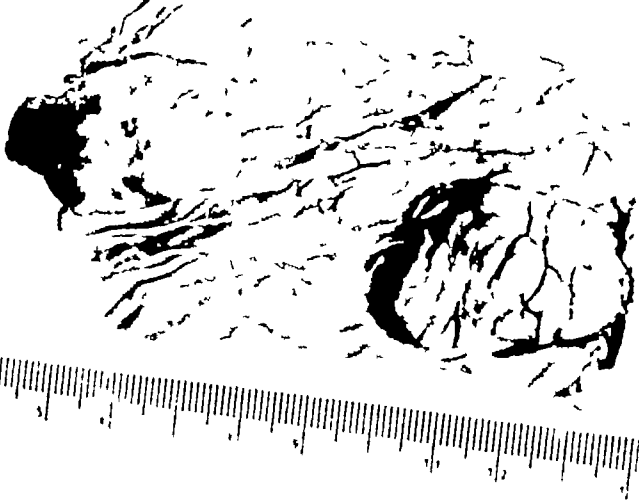
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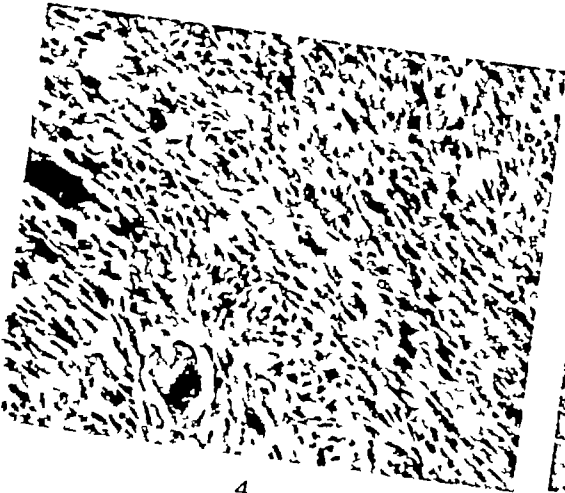
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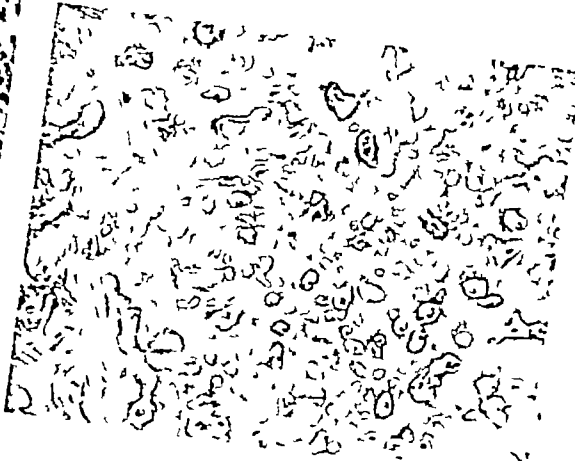
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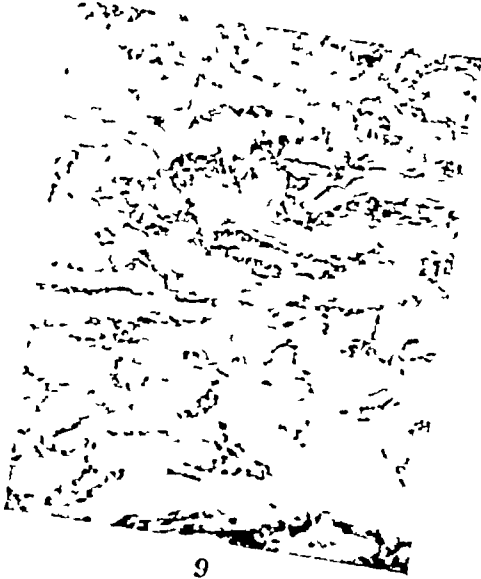
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But although the histogenesis of osteogenic sarcoma of soft tissues can be readily explained along these lines, they are uncommon tumours and as such worthy of note

SUMMARY

A case of osteogenic sarcoma of the extraskeletal soft tissues of the loin is recorded, with widespread bone-forming metastases studied at necropsy

The histogenesis of this type of growth is discussed and it is concluded that these tumours arise due to aberrant differentiation of the multiplying malignant mesenchymal cell

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3 4-BENZPYRENE IN INDUSTRIAL AIR POLLUTION SOME REFLEXIONS

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AMONG the known carcinogens producing lung carcinoma in man arsenic, asbestos, beryllium, chromium, nickel and ionizing agents can account for only a small fraction of all cases occurring, because these agents are very limited in their distributions

As 3 4-benzpyrene, a well known experimental carcinogen for epidermis and connective tissue, has been found in tobacco smoke (Cooper, Lindsay and Waller, 1954, Cooper and Lindsay, 1955, Seelkopf, 1955) and in the air of populated areas, especially town air (Waller, 1952, Kotin, Falk, Mader and Thomas, 1954, Stocks and Campbell, 1955, Campbell and Clemmesen, 1956, Campbell and Kreyberg, 1956), the view has been advanced by numerous students, that 3 4-benzpyrene is the important factor in the increase in lung carcinomas in recent years, especially among males in towns

Stocks and Campbell in their paper even attempted to calculate the respective role of 3 4-benzpyrene from the two sources Such calculations are, however, of restricted interest because the cases of lung tumours used for the calculations were mainly based upon death certificates and the histological typing ignored The latter point is of special importance when the absolute number of cases is low, as is the case in rural districts in Norway (Kreyberg, 1959) It may be of considerably lesser importance in England and Wales, where the lung cancer incidence is high, even in rural districts

In the rural areas not only the amount of 3 4-benzpyrene in the general air is lower than in more densely populated areas and towns, but the number of special factories and industrial plants producing carcinogens are also fewer A certain consideration should therefore be paid, when lung cancer frequency in urban and rural areas are compared, to the occupation of the victims

It may be that some of the lung cancer patients in the rural districts have been exposed to important concentrations of carcinogens during their working hours, in spite of living in an air little polluted generally With the increasing dispersion of industry in countries with ample access to water-power (and in the future to atomic power) an increasing population will work in factories with polluted air, in areas administratively designated as " rural "

Under these circumstances a study of the occurrence of 3 4-benzpyrene in industrial plants should be of some interest

Gasworks naturally present themselves for study, because gas fumes contain benzpyrene, and statistical evidence points in the direction that lung cancer occurs more frequently among gas workers, than in the general population (Kuroda and Kawahata, 1936, Kennaway and Kennaway, 1947, Doll, 1952)

Another source of air, polluted with 3 4-benzpyrene, came to my attention some years ago, when a paper by Livingston (1953) described warts appearing on the hands, arms, ankles and face of workers connected with the Søderberg furnace in an aluminium factory in England

At the same time I had the opportunity to examine a tar developed during aluminium production in a Norwegian factory The tar showed a very high carcinogenic potency when painted on the skin of mice, and by chemical analysis the tar was shown to contain 1 per cent 3 4-benzpyrene

During the aluminium production fumes from such tars will in varying amount pollute the air of some parts of the factory

On this background I sought contact with the gasworks of Bergen (director B Paulson) and Oslo (director W Eckhoff), as well as with the Norwegian State-owned and controlled aluminium factories (director Aa W Owe)

The two directors of the gasworks readily gave their full support The director of the aluminium factories, however, flatly refused co-operation on similar terms My application for permission to study the air in the privately owned A/S Elektrokemisk, which holds the rights of the Søderberg electrode was, however, immediately generously granted by the director-general G Hagerup Larssen, and the director of Fiskaa Verk J Gørrissen, and all facilities extended

Gasmeters equipped with filter papers were installed in the plants at representative sites where the workers would be regularly employed, as described in a previous paper (Campbell and Kreyberg, 1956)

The filter papers were most kindly examined by Mr B T Commins at the " Group for Research on Atmospheric Pollution ", St Bartholomew's Hospital, London

The results of the investigations of the three industrial plants are summarized in Table I For comparison figures from the air of some towns in England and Wales are given, quoted from Commins (1958)

TABLE I —*The Content of Hydrocarbons in the Air in µg per 100 m³*

	Source of sample					
	Industrial Plants			General Town Air		
Hydrocarbon	Oslo Gasworks	Bergen Gasworks	Elektrokemisk (µg per 100 m³)	Liverpool	Wrexham	Llangefni
Fluoranthene	1300	240	100	6 7	2 0	0 4
1 2 benzanthracene	1400	280	80			
Phenanthrene	Not detected	s	27			
3 4 benzpyrene	150	130	10			
Pyrene	870	240	60	5 0	1 8	0 3
Coronene	20	50	Trace			
1 12 benzperylene	210	200	5	16 6	5 1	0 5
3 4 Benzfluoranthene	730	200	18	6 8	2 0	0 4
Anthracene	100	Trace	Present		0 4	0 1
Anthanthrene	80	60	Trace			
Fluorene	20	Trace				
Naphthalene	Not detected	Not detected				

For further comparison, the seasonal changes of 3 4-benzpyrene in the air in towns of England and Wales and in Norway are presented in Fig 1, quoted from the paper of Campbell and Kreyberg (1956)

The first comment to these findings is to stress that they do not at all intend to give a complete picture of the air pollution in such plants   The report actually represents a pilot study and should be followed up with more extensive and more complete investigations
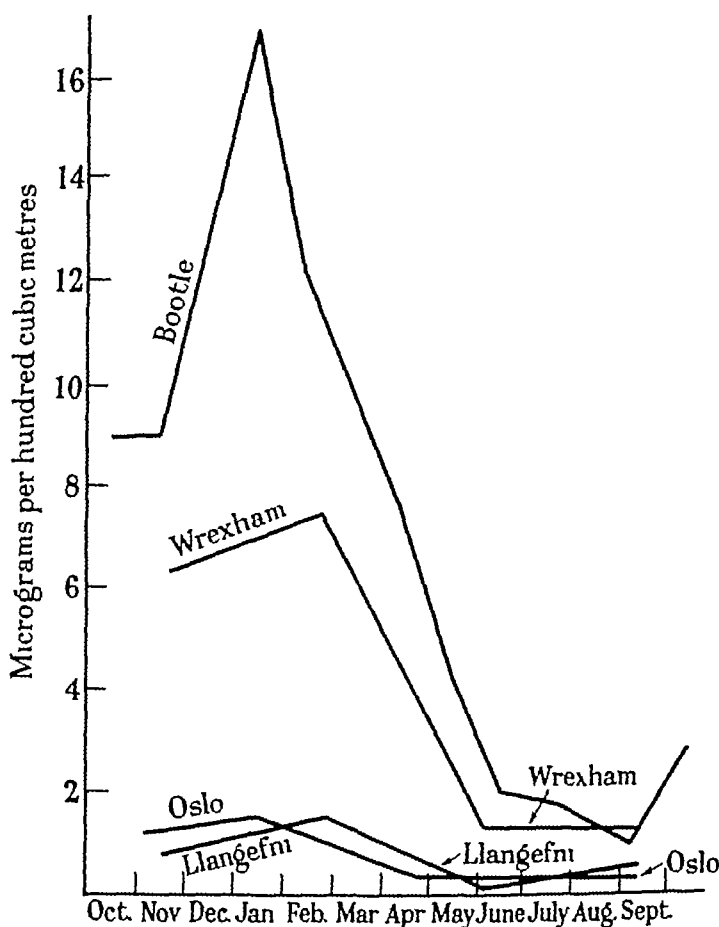
Fig 1 —Yearly concentration of 3 4 benzpyrene in the air at Oslo in 1955 compared with Bootle, Wrexham and Llangefni in England and Wales (Campbell and Kreyberg, 1956)

With the coarse technique used, the figures indicate that the air in the retort house of Oslo Gaswork contains 3 4-benzpyrene in amounts corresponding to some 5000 cigarettes daily for a worker with a 40 hours week   Even if the figures for Bergen are lower, they are nevertheless of the same order of magnitude and the " cigarette-equivalent " is enormous

From the study of cases of human lung cancer two observations are of interest in this connection   1 There seems to be a straight line correlation between the number of cigarettes smoked and the risk of developing epidermoid and small cell anaplastic carcinomas   In an English material the increase in risk is some 25 times after regular daily smoking of 30 cigarettes (Doll, Hill and Kreyberg, 1957)   2 The risk among gas workers to develop lung cancer is in the literature

(Doll, 1952) ranged between twice and ten times the risk of the general population, smokers included

If these two facts are correlated   the enormous amounts of 3   4-benzpyrene in the air and the very moderate excess of lung cancer in the gasworks, a serious fallacy is evidently involved   One explanation of the discrepancy between amount of 3   4-benzpyrene in the air and risk of lung cancer may be the following

The benzpyrene is suspended in the air as finer or coarser particles adsorbed to other particles, particularly flakes of soot   Biologically active is only that fraction which enters the body, in the present case the bronchial linings

Besides, the physico-chemical measurement of the substance under suspicion, also knowledge of the particle size is necessary, as only particles under and above a certain size are deposited in any numbers on the bronchial linings

This represents a serious warning against any conclusions as to causative relationships between any substance and lung cancer based upon the mere finding of the substance deposited on paper filtering the air

Another explanation of the lack of correspondence between the amounts of 3   4-benzpyrene in the air and the number of lung cancer cases in the gasworks may be that there is no causative relationship at all

The overwhelming evidence to-day shows that the rise in frequency in epidermoid and small cell anaplastic lung carcinomas is mainly caused by cigarette smoking   The active agent in the cigarette smoke is not known

The main support for the assumption that 3   4-benzpyrene is an important factor in the increased development of lung cancer is its immediate plausibility   The substance is present in polluted town air and in tobacco smoke, and the substance is carcinogenic in animals

But 3   4-benzpyrene is present in cigar and pipe smoke in even greater concentrations than in cigarette smoke (Cardon, Alvord, Rand and Hitchcock, 1956, Gilbert and Lindsay, 1956), but not connected with anything like the same risk for lung cancer development   The role of 3   4-benzpyrene in the development of lung cancer is very far from known

The main purpose of publishing these sparse data is to enter a plea for a more complete technique for future studies of possible carcinogens in the air inhaled by the human population   The particle size should be recorded in addition to the statements of the amounts present

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INCOMPLETE CARCINOGENS IN CIGARETTE SMOKE CONDEN-SATE TUMOUR-PROMOTION BY A PHENOLIC FRACTION

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With an Appendix by J G BURGAN

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The carcinogenicity of tobacco smoke condensate for mouse skin, which was demonstrated by Wynder and his colleagues in 1953 and subsequently (Wynder, Graham and Cronninger, 1953 , and Wynder, 1959 for review), has now been confirmed by several other groups of workers (Engelbreth-Holm and Ahlmann, 1957 , Orris et al , 1958 , and Bock and Moore, 1959) Passey (1957) also observed benign and malignant skin tumours in mice painted with tobacco smoke condensate He pointed out, however, that the mice, which were of one of the strains used by Wynder and his colleagues, developed spontaneous ulcers of the skin These appeared in untreated and treated mice alike, and the author considered that their occurrence rendered interpretation of the results difficult In all these experiments the smoke condensate was applied at short intervals for long periods

The possibility that the two stage mechanism of carcinogenesis might be involved was early considered Wynder et al (1953) included an experiment in which tobacco smoke condensate was applied together with the tumour-promoting agent, croton oil to mouse skin The yield of tumours was no greater than with smoke condensate alone Gwynn and Salaman (1956) examined whole smoke condensate for the presence of incomplete carcinogens They tested the condensate for both tumour-initiating and tumour-promoting activity, and obtained some evidence of promotion but none of initiation The results of Hamer and Wood-house (1956) also suggested that smoke condensate has tumour-promoting action, but they obtained no evidence for tumour initiation, or for complete carcino-genicity Gellhorn (1958), in several large scale experiments, demonstrated very convincingly the tumour-promoting effect of whole smoke condensate

Wynder at a meeting at the Chester Beatty Research Institute on July 6, 1959 spoke of evidence which his group had obtained for tumour-promotion by the nicotine-free basic fraction of smoke condensate

The experiments to be described are a further stage in the examination of tobacco smoke condensate for incomplete carcinogens (tumour initiators and promoters) The strong promoting action of one fraction of the condensate is reported

MATERIALS AND METHODS

Mice —Male and female mice of the " 101 " inbred strain were used   They have been maintained by brother-sister mating in this department since a breeding pan was obtained from Dr T C Carter (Radiobiological Research Unit, A E R E , Harwell Didcot Berks) in November, 1955

This strain was selected for its high and uniform susceptibility to skin tumour-induction by initiating and promoting agents (Salaman, 1956)   Not until half way through the experiments was it realized that the mice are not very suitable for long term experiments because of a high incidence of a renal disease, papillo-nephritis first observed in Strain A mice by Gorer (1940), and subsequently studied in greater detail by Dunn (1944)   As in strain A mice, the disease shortens the life span of affected animals so that few live as long as 18 months

Mice were fed on cubes according to the Rowett Institute formula (Thomson, 1936) and water *ad libitum*

Before the experiment mice were vaccinated on the tail with sheep lymph as a precaution against ectromelia   all responded with a typical primary response

The dorsal hair was removed by electric clippers at the beginning of the experiment and thereafter as necessary

Preparation of cigarette smoke condensate and its fractions

These were kindly supplied by the Research Laboratories of the Tobacco Manufacturers' Standing Committee

Details of the preparations are given by Mr J G Burgan in an appendix to this paper

Freshly prepared samples of whole smoke condensate and its neutral fraction were mailed to us each week, and were used within two weeks of receipt   Storage between arrival and use was in small sealed ampoules kept protected from light at 4° C

Large samples of the smoke phenol fraction were mailed to us at 6-weekly intervals and stored similarly before use

Application of materials to the skin

9 10-Dimethyl-1 2-benzanthracene (DMBA), obtained from L Light & Co , was applied as a 0 15 per cent solution in acetone with a graduated pipette

All the smoke condensate fractions were too viscous, even after dilution with acetone, to be applied quantitatively with a graduated pipette, or by a wide-mouthed dropper   a brushing-on technique was necessary   The whole of the back from the neck to the root of the tail was painted, using a squirrel-hair brush

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EXPLANATION OF PLATE

Fic   1 —Papillomata on the back of a mouse treated once with DMBA (300 μg) and then thrice weekly with the phenolic fraction of cigarette smoke condensate (6–12 mg per application) for 27 weeks

Fic   2 —Photomicrograph of a malignant tumour which arose on the dorsal skin of a mouse similarly treated with phenolic fraction for 41 weeks after a single application of DMBA   Invasion of the panniculus carnosus muscle can be seen   (Staining   haematoxylin and eosin   Magnification   × 230)

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The whole smoke condensate and neutral fractions as received were diluted 3 or 4 times with acetone After each painting session the average amount of condensate or fraction applied to each mouse was calculated retrospectively as follows —

$$\text{Average weight of material applied to each mouse} = \frac{\text{Wt of fraction} + \text{acetone used}}{\text{No of mice painted}} \times \text{per cent fraction in fraction-acetone mixture}$$

With experience it was possible to keep this average to within $\pm$ 20 per cent of the intended dose

The smoke phenol fraction was diluted with acetone 1 in 4, 1 in 6, or 1 in 8 according to dose level It was found that painting the clipped skin with the strongest of these concentrations left on the average 12 mg of smoke phenols on the mouse , 1 in 6 left 9 mg , and 1 in 8 left 6 mg

The acetone used was of the Analar grade supplied by British Drug Houses

Histological examination—Biopsy specimens, and tissues taken for section post mortem, were fixed in Zenker's fluid, embedded in paraffin wax, and stained with haematoxylin and eosin

EXPERIMENTAL

Experimental I   whole smoke condensate

Thirty male and 32 female mice were clipped, and painted thrice weekly with whole smoke condensate At first approximately 25 mg was applied to each mouse at each application This dose was steadily increased during the first 3 weeks up to an average of 40 mg per mouse, and thereafter maintained at approximately this level until the 30th week It was then reduced, because of toxic effects, to 30 mg per application

Table I indicates the progress of the experiment Fifty-two mice died during the first 40 weeks   gross pathological changes indicative of papillonephritis (see p 624) were present in 13 mice (this incidence does not differ significantly from that in untreated mice), pneumonic consolidation in 6, intussusception in 3, enteritis in 3 Nineteen mice died during the 24th week from the toxic effects of an unusually large dose of smoke condensate applied in error Several other mice which showed no obvious lesions, and some of those with only slight kidney lesions, probably died from the cumulative toxic effects of the condensate Advanced post mortem changes prevented satisfactory examination in 11 mice

One papilloma appeared during the 37th week, when there were only 13 survivors The same mouse developed a second papilloma during the 44th week, but died two weeks later No other tumours have appeared There are still 3 mice alive after 60 weeks of treatment but none has any skin tumours

Experiment II   Neutral fraction

Thirty male and 33 female mice were clipped and painted thrice weekly with the neutral fraction of tobacco smoke At first approximately 25 mg was applied to each mouse at each application This dose was steadily increased during the first 3 weeks up to an average of 40 mg per mouse This level was thereafter maintained

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TABLE I —*Tests of Whole Smoke Condensate and its Neutral Fraction for Carcinogenicity on Mouse Skin*

Time (in weeks)	Total weight of test material applied* (g)	Survivors	Number of mice with papillomas	Total papillomas	Total carcinomas
		Experiment I Whole Smoke Condensate			
0	—	62	—	—	—
5	0 50	62	—	—	—
10	1 10	62	—	—	—
15	1 66	59	—	—	—
20	2 22	50	—	—	—
25	2 72	30	—	—	—
30	3 17	22	—	—	—
35	3 52	13	1	1	—
40	3 96	11	2	2	0
		Experiment II Neutral Fraction			
0	—	63	—	—	—
5	0 50	60	—	—	—
10	1 10	59	—	—	—
15	1 66	58	—	—	—
20	2 22	46	—	—	—
25	2 78	44	—	—	—
30	3 38	37	—	—	—
35	3 90	25	3	4	—
40	4 46	17	1	1	0

* In both experiments the test material was applied thrice weekly throughout   At first 25 mg amounts, diluted 3 or 4 times in acetone, were applied on each occasion   This dose was steadily increased to 40 mg per application during the first 3 weeks of treatment   In the case of the neutral fraction the dose level was maintained at 40 mg throughout the experiment, but in the case of whole smoke condensate it was reduced to 30 mg per application after 30 weeks because of systemic toxicity

Table I indicates the progress of Experiment II   Thirty-eight mice died during the first 40 weeks   Compared with the mice treated with whole smoke condensate, pneumonic consolidation (12 cases) was more frequent in these mice   Tyzzer's disease caused 3 deaths, intussusception 3, and enteritis a further 4   Papillonephritis was seen in 18 mice   One mouse died as a result of haemorrhage from a tumour of the tongue   On histological examination this tumour was of squamous cell type, it showed doubtful invasion of muscle and no metastases It was regarded as probably, but not certainly, malignant   Advanced post mortem changes prevented satisfactory examination in 10 mice

Two papillomas appeared during the 32nd week of the experiment, one in a mouse which died 10 days later (post mortem decay prevented satisfactory examination), and the other in a mouse which died 4 weeks later and showed marked renal changes due to papillonephritis   Whilst under observation, both tumours were notably slow-growing   A third mouse developed a papilloma during the 40th week of the experiment   Treatment was stopped after 47 weeks   Three weeks later two of the 10 survivors (both females) developed 2 papillomas each These grew more rapidly than those which arose earlier, and soon one of the tumours on each mouse took on the naked-eye appearances of malignancy   One was removed at biopsy during the 60th week and was found to be a squamous-cell carcinoma invading muscle

Experiment III   Smoke phenols fraction

One hundred and ten mice, divided into 3 groups, were used in this test Each group contained equal numbers of males and females   Groups I and II consisted of 30 mice each, and Group III of 50 mice   Groups I and III were given a single application of 0 2 ml   0 15 per cent DMBA in acetone to the clipped dorsal skin   Group II was not treated at this time   Three weeks later groups I and II began a course of thrice weekly applications of smoke phenols fraction in acetone   For the first 9 applications a 25 per cent solution/suspension of the fraction in acetone (i e average of 12 mg smoke phenols per mouse per application) were given   The skins of many animals became thick and crusted, and 3 mice died on the 19th day as a result of systemic toxicity   The concentration was therefore reduced to 12 5 per cent and continued at this level for a further 15 applications   It was then increased to 18 7 per cent for 24 applications, and then back to 25 per cent from the 49th application (17th week) onwards   Group III received no treatment apart from the single application of DMBA

Of the 10 mice of Group I which died during the first 40 weeks of the experiment, i e 37 weeks of treatment with smoke phenol fraction, one had lymphatic leukaemia with enlargement of the thymus and lymph glands, one had a large mesenteric tumour (unfortunately post mortem changes were too advanced for satisfactory histological examination) 2 showed pneumonic consolidation, and 1 had 2 small lung adenomas   Four had abscesses in the liver and 3 showed papillonephritis

Of Group II, 14 mice died   As mentioned above, 3 died early (19th day) from systemic toxic effects   A large abdominal tumour was seen in a female mouse which died during the 37th week   This tumour was adherent to the spleen, pancreas and mesentery   Histological examination showed it to be a haemangioma This mouse was one of 3 which had liver abscesses   Three mice showed pneumonic consolidation, and 7 had papillonephritis

In Group III 13 mice died and 7 of these were examined post mortem   One had lymphatic leukaemia with a grossly enlarged thymus and one had 2 small lung adenomas   One mouse had intussusception, one bronchopneumonia, and one a large abscess of the neck   Five of the 7 showed kidney lesions indicative of papillonephritis   Advanced post mortem changes prevented satisfactory examination of the remaining 6 mice

Incidence of skin tumours

Papillomas began to arise in mice of Group I during the 15th week   By the 37th week of treatment with the phenolic fraction 15 out of the 20 survivors of this group bore a total of 65 papillomas (see Table II and Fig 1)   All these tumours arose on the treated area of skin, and none were seen outside it   At the 37th week one tumour on a male showed macroscopic features suggestive of malignancy During the next 4 weeks this tumour grew quickly, ulcerated, and became adherent to deeper structures   It was removed by biopsy during the 41st week of the experiment, and histological examination showed it to be a poorly differentiated carcinoma which had penetrated the panniculus carnosus muscle (Fig 2)

Treatment with smoke phenol fraction was stopped after 41 weeks (44th week of experiment)   The mice have been kept under observation , a further probably malignant tumour has arisen on another mouse (a female) but no specimen has yet been taken for histological examination

TABLE II.—*Tests of the Phenolic Fraction of Cigarette Smoke Condensate for Carcinogenicity, and for Tumour-promotion after a Single Application of DMBA, on Mouse Skin*

Group	Number of mice	Treatment	Tumour incidence at 40 weeks*			
			Survivors	Mice with papillomas	Total papillomas	Total carcinomas
I	30	300 µg DMBA followed, after 3 weeks interval, by thrice weekly applications of smoke phenols fraction†	20	15	65	1
II	30	Thrice weekly applications of smoke phenols fraction only	16	0	0	0
III	50	300 µg DMBA only	37	4	6‡	0

* i e After 37 weeks of smoke phenols treatment

† The smoke phenols fraction was diluted with acetone 1 4 to 1 8, depending on dose level (see text, p 625) The first 9 applications of smoke phenols fraction were of 12 mg each, the next 15 were of 6 mg, the next 24 of 9 mg, and thereafter all applications were of 12 mg each

‡ All outside the treated area (see text, p 000)

No tumours have appeared on mice of Group II

Four mice in Group III developed a total of 6 papillomas during the 40 weeks after treatment with DMBA All these tumours were situated on the head no tumours arose on the treated dorsal skin It is odd that no such ectopic tumours arose in Group I mice which received similar treatment with DMBA as well as treatment with smoke phenols fraction A similar result was obtained in this laboratory, in " S " strain mice treatment with DMBA alone gave rise to many ectopic tumours, whereas DMBA followed by repeated applications of croton oil gave rise to very few (Roe, 1956)

Whatever the explanation of this curious phenomenon, the tumour-incidence in Group I is significantly higher than that in Groups II and III, and the results clearly indicate that under the experimental conditions described the phenolic fraction of cigarette smoke condensate has considerable tumour-promoting activity but no carcinogenic activity

DISCUSSION

Recent reports (Engelbreth-Holm and Ahlmann, 1957, Orris et al, 1958, and Bock and Moore, 1959) have confirmed the findings of Wynder and his colleagues (Wynder, Graham, and Croninger, 1953, and see Wynder, 1959, for review), and the view is becoming generally accepted that cigarette smoke condensate is carcinogenic for mouse skin when applied at short intervals for long periods The results of Experiments I and II in which the whole smoke condensate and its neutral fraction were tested in this way were unsatisfactory because of poor survival of mice a few benign tumours arose in both experiments after 35 weeks of continuous treatment, and 2 malignant tumours at a much later date in the neutral fraction experiment

Much attention has been given to the identification of the carcinogenic components in tobacco smoke condensate Kennaway and Lindsey (1958) give a long list of individual substances which have been identified in smoke, and a short

list of 10 for which there is at present evidence of carcinogenicity Most attention has been paid to the 3,4-benzpyrene content Cooper, Lindsey and Waller (1954), Latarjet et al (1956), and Bentley and Burgan (1958) all reported amounts of the order of 1 μg of 3,4-benzpyrene in the smoke from 100 g cigarettes Wynder and Wright (1957) and Wynder and Hoffmann (1959) found slightly larger amounts than this, namely, about 4 μg per 100 g cigarettes Cardon et al (1956) on the other hand, estimated the benzpyrene, yield from 100 g cigarettes at between 10 and 12 μg, but the calculations on which these higher estimates are based have been criticised by Bentley and Burgan (1958) Whichever estimate is correct it is very doubtful whether the amount of benzpyrene in smoke condensate is sufficient to account for the tumours seen in experiments with mice Approximately 4 g of condensate are produced by smoking 100 cigarettes (of 1 g each), the amount varying according to the smoking conditions used If we suppose that the higher figure given by Cardon et al is correct, 4 g of condensate would contain 12 μg of 3,4-benzpyrene, and 40 mg of condensate (the amount applied thrice weekly by Wynder and his colleagues) would contain approximately 0 1 μg If, on the other hand, the figures given by Cooper, Lindsey and Waller, Latarjet et al , and Bentley and Burgan is correct, the amount of 3, 4-benzpyrene per application would be < 0 01 μg Poel and his colleagues (see Poel, 1959) applied 0 38 μg 3,4-benzpyrene to mice thrice weekly two carcinomas (after 82 and 83 weeks respectively) and 9 papillomas (after an average of 69 weeks) arose in a group of 55 C57 mice The same workers produced 5 papillomas (average induction time = 60 weeks), but no malignant tumours, in a similar group of mice painted thrice weekly with 0 15 μg 3,4-benzpyrene Even allowing for different experimental techniques and the strains of mice used, it seems most unlikely that the carcinogenic effect of smoke condensate observed by Wynder's group, and by others including ourselves, is due to 3, 4-benzpyrene alone

Estimates of the amounts of the other carcinogens listed by Kennaway and Lindsey (1958) are either not available or highly conjectural But, taking into account all the available data, and in particular the fact that several of the other carcinogens present are much weaker than 3,4-benzpyrene, it is unlikely that their carcinogenic effect combined with that of 3,4-benzpyrene could equal that of the whole smoke condensate Dr Wynder expressed the same view at a meeting held at the Chester Beatty Research Institute in July 1959 Of course it may be argued that since several of the substances listed by Kennaway and Lindsey have not been tested for carcinogenic activity, their possible contribution cannot be assessed But to assume that their concentration and potency are sufficient to add appreciably to the carcinogenic effect of the benzpyrene and the other known carcinogens is surely inadmissible at this stage

Turning to the possible role of incomplete carcinogens in tumour production by cigarette smoke condensate, the fact that a substance is carcinogenic when applied alone may be taken to indicate that it has both tumour-initiating and tumour-promoting properties However, amounts of carcinogenic hydrocarbons too small to give detectable carcinogenic effect, e g 1 μg 9-10-dimethyl-1, 2-benzanthracene (DMBA) (Klein, 1956) have demonstrable initiating action Total doses of 3,4-benzpyrene of this order were undoubtedly applied in the course of the applications of smoke condensate by Wynder and others Thus these amounts of condensate contained enough 3,4-benzpyrene for the initiation of the tumours

which arose, though certainly not enough for their production without the aid of a tumour promoter Furthermore, in addition to the small amounts of 3,4-benzpyrene and other complete carcinogens, there may also be present in cigarette smoke condensate incomplete carcinogens of the type of which urethane and triethylene melamine are examples (Salaman and Roe, 1953 , Roe and Salaman, 1955), i e substances with initiating but no promoting action Little is known of the distribution of such substances, and their presence in tobacco smoke condensate has still to be demonstrated

Thus on quantitative grounds alone we would expect to find tumour-promoting substances in cigarette smoke condensate Experiment III reported here is a clear demonstration of tumour-promotion by the phenolic fraction, constituting about 15–20 per cent of the condensate Commins and Lindsey (1956) estimated quantitatively some of the simpler phenols present in cigarette smoke, and recorded the total yields of 9 different phenolic compounds in micrograms per cigarette The yield of phenol itself was 123 μg per cigarette, quinol—83 μg , catechol—61 μg , and *ortho meta*, and *para*-cresol, together—80 μg Resorcinol, 1-naphthol, and 2-naphthol were present in much lower quantities The total yield of all 9 compounds amounted to 356 μg per cigarette, which is equivalent to 0 36 g per 1,000 cigarettes The yields of phenol and *ortho*-cresol reported by Commins and Lindsey were close to those previously recorded by Rayburn, Harlan and Hanmer (1953)

In the experiments reported here the total yield of phenol fraction from 1,000 cigarettes was 4 8 g (see Appendix) Thus the substances studied by Commins and Lindsey (1956) account for less than one tenth of the phenol fraction used in Experiment III, and the composition of the remaining nine tenths is still not known

In view of the known tumour-promoting activity of phenol and related compounds, (Boutwell, Rusch and Bosch, 1955 , Salaman and Glendenning, 1957 , Boutwell and Bosch, 1959) the result of Experiment III is perhaps not entirely unexpected Quantitatively the tumour-promoting effect observed here was comparable to that for whole smoke condensate observed by Gellhorn (1958), i e the promoting activity of the phenolic fraction could have accounted for all the effect observed by Gellhorn However differences in methods and strain of mice make such a quantitative comparison unwise

If we assume that the carcinogenic effect of smoke condensate is due to the action of initiators (known or unknown) plus promoters (phenols and others), it is on the face of it difficult to explain why tests of the condensate for initiating action, by the simultaneous and/or subsequent application of croton oil, gave no tumours Certainly promotion was more than adequate Was enough initiator applied in these tests ? In the experiments of Gwynn and Salaman (1956) a total of about 1 5 g of smoke condensate was applied as an initiating dose The benzpyrene content of this according to Cooper *et al* (1954) and Bentley and Burgan (1958) would have been 0 3 μg , and according to Cardon *et al* (1956) about 4 μg This is of the order of the minimum initiating dose of DMBA reported by Klein (1956) Hamer and Woodhouse applied only about 0 2 g of smoke condensate in their test for tumour-initiation, which would contain an amount of benzpyrene definitely below the detectable initiating dose Thus it is probable that in the experiments specifically designed to show initiating action, too little initiator was applied

We may sum up the present position with respect to tests on mouse skin by saying that —

(1) the observed tumour producing action of cigarette smoke condensate is unlikely to be due to contained substances acting as complete carcinogens,

(2) it can be explained as due to the combined action of tumour initiators and promoters, and,

(3) since its effect can be increased by the addition of initiators but not of promoters, the relative weakness of the carcinogenic action of cigarette smoke condensate is due to the paucity of initiators

There is at present no direct evidence for two-stage carcinogenesis in human bronchial epithelium Assuming however that this mechanism operates here, as it has been shown to do in skin and some other tissues, present evidence suggests that smoking has stronger tumour-promoting than tumour-initiating effect

Man, whether a smoker or not, is exposed to substances of proved tumour-initiating power in the polluted atmosphere, and in the products of some industrial processes and in some kinds of food The correlation between smoking habits and lung tumour incidence may well be determined not primarily by the carcinogenic effect of tobacco smoke but by its predominantly tumour-promoting action on the bronchial epithelium

SUMMARY

Strong tumour-promoting effect by a phenolic fraction of cigarette smoke condensate applied after a single tumour-initiating dose of 9,10-dimethyl-1,2-benzanthracene (DMBA) to the dorsal skin of " 101 " strain mice was observed sixty-five benign and 2 malignant tumours arose on 30 treated mice during 40 weeks of treatment The same dose of DMBA alone produced a negligible number of tumours, and the phenolic fraction alone produced none

" 101 " Strain mice treated with whole smoke condensate only, or the neutral fraction of the condensate only, developed a few papillomas after 35 weeks However, the survival of the mice was poor, and very few were alive at the time when malignant tumours might have been expected only two were seen One of the reasons for the poor survival was a renal disease, papillonephritis, previously recorded in strain A mice

Reasons are given for believing that cigarette smoke is richer in tumour-promoting than in tumour-initiating substances Phenolic compounds may be responsible for much of its promoting activity

The implications of these findings, and their relevance to the induction of bronchial carcinoma in man, are briefly discussed

APPENDIX   THE PRODUCTION OF CIGARETTE SMOKE CONDENSATE

(by J G Burgan, Research Laboratories, Tobacco Manufacturers' Standing Committee)

Smoking technique

Cigarettes, a mixture of brands popular in the United Kingdom, are smoked in batches of 1,000 using an automatic smoking machine of the type described by Iles and Sharman (1957), arranged to give a puff frequency of 4 per minute, a puff volume of 15 ml, and a puff duration of 2 seconds   Cigarettes are smoked to a stub length of 20 mm   The smoke condensate is collected in traps immersed in a dry ice/acetone mixture, one trap being attached to each smoking position   The type of cold trap used has been described by Burgan (1959)

Whole smoke condensate

For the production of whole smoke condensate the material collected in the cold traps is washed out with acetone   The solvent is then removed on the water-bath and the whole smoke so obtained is stored in the dark in sealed ampoules at $-25°$ C

Phenolic and neutral fractions

For the separation of smoke condensate into its main fractions the material from 1,000 cigarettes, collected in the cold traps, is dissolved in a mixture (1   3 v/v) of hydrochloric acid (2N) and ether (peroxide free), the ether used having been previously treated with sodium wire to remove fluorescent material   The aqueous acidic layer is separated and the ether layer washed further with portions of hydrochloric acid (2N, 4 × 100 ml) to complete the removal of basic compounds

The ether layer is then washed with portions of saturated sodium bicarbonate solution (1 × 200 ml, 4 × 100 ml) to remove carboxylic acids, and with portions of 3 per cent potassium hydroxide solution (1 × 200 ml, 5 × 100 ml) to remove any carboxylic acids remaining together with the phenols present

The residual ether layer containing the neutral fraction of smoke condensate is dried over anhydrous magnesium sulphate and the solvent removed on the water-bath   The neutral fraction obtained is stored in the dark in sealed ampoules at $-25°C$

The phenols are recovered from the combined potassium hydroxide washings These are neutralized with hydrochloric acid (2N), excess sodium bicarbonate (solid) added, and the mixture saturated with sodium chloride and extracted with portions of ether (1 × 200 ml, 4 × 100 ml)   The combined ether washings, containing the phenols, are dried over anhydrous magnesium sulphate and the solvent removed on the water-bath   The phenols obtained are stored in the dark in sealed ampoules at $-25°$ C

The average amounts of whole smoke, phenols, and neutral fraction obtained from 1,000 cigarettes, using the smoking conditions described above, are respectively 29 5 g, 4 8 g and 16 6 g

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TUMOUR - INHIBITING ACTION OF 1,6 - Di - (2 - BROMOETHYL - AMINO) - 1,6 - DIDEOXY - D - MANNITOL DIHYDROBROMIDE [DBM (R13)]

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NITROGEN mustard is at present the agent outstanding in the chemotherapy of malignant tumours   It furnishes the basis for most of the drugs prepared since Gilman and Philips (1946) synthesised the compound, by substituting a sulphide group for the amino group of mustard gas, and found it developed remissions of transplantable mouse lymphosarcoma   While the results it has yielded in Hodgkin's disease, lymphosarcoma, and chronic leukaemias, must not be undervalued (Goodman *et al*, 1946, Jacobson *et al*, 1946, Karnofsky, 1950), nitrogen mustard has proved to be of no value in the treatment of carcinomatous tumours

Nitromin, prepared by Stahmann and Bergmann (1946) was the first nitrogen-mustard derivative to have a more marked growth-inhibiting effect on Yoshida sarcoma than nitrogen mustard itself, and to be of lesser toxicity (Ishidate, Kobayashi, Sakurai, Sato and Yoshida, *cit* Farber *et al*, 1956)   In Japan, Nitromin is being widely used in human therapy   Kimura and his co-workers (*cit* Farber *et al*, 1956) found that in addition to being of avail in cases of chronic leukaemia, it had a destructive effect on certain malignant tumours

Another nitrogen-mustard derivative is Erysan (R48), an aromatic chloroethylamine, first synthesised by Haddow and his co-workers (1948), which was shown to be highly effective on the Walker carcinosarcoma   Matthews (1950) reported its effectiveness in chronic myeloid and lymphoid leukaemia in humans, and Videbaek and Kaae (1954) in such cases of Hodgkin's disease as had failed to respond to X-ray treatment

Everett, Roberts, and Ross (1953) synthesised N,N-di-(2-chloroethyl)-*p*-aminophenyl-butyric acid, an aromatic nitrogen-mustard derivative, which they labelled CB 1348   This compound was found considerably to inhibit the growth of Walker tumours and according to Haddow (1954), to have a beneficial effect on lymphoreticular tumours and haemoblastoses, its depressant effect on bone marrow was less than that of nitrogen mustard and TEM, respectively

Larionov (1957) reported good results obtained with Sarcolysin and Dopan Simultaneously with, but independently of Larionov and co-workers (1955), Bergel and Stock (1954) also prepared sarcolysine dl-*p*-di(chloroethyl)-aminophenylalanine hydrochloride   It caused complete regression in two sarcomas of the rat and one of the mouse, and had a marked inhibitory effect on the growth of seven other mouse and rat tumours

For some years Vargha has endeavoured to produce an agent of reduced toxicity, by linking nitrogen mustard with a sugar   Of the many compounds

he has prepared 1,6-di-(2-chloroethylamino)-1,6-dideoxy-D-mannitol dihydrochloride has proved to be of good effect   Having been investigated in animal experiments by Kellner and Németh (1956) and tested clinically by Sellei and Eckhardt (1958), it has been introduced in human therapy as Degranol (BCM)

MATERIALS AND METHODS

Twenty-three combinations of nitrogen mustard with sugar, prepared by Vargha in the course of the last two years, were tested for their respective toxicity, and studied in transplantable animal tumours for their growth-inhibiting action Of these, one compound, marked R_{13}, the structural formula of which may be represented as 1,6-di-(2-bromoethylamino)-1,6-dideoxy-D-mannitol dihydrobromide (DBM), was found to be the most promising (Vargha and Horváth, 1959 , Baló, Kendrey, Juhász, Besznyák, 1959)   This is a stable compound readily soluble in water, of which 23 mg /kg of body weight is the half-lethal dose (LD_{50}) for the mouse, and 22 mg /kg that for the rat

Using Wistar strain rats and white mice of our own breed, experimentation in normal intact animals and animals with transplanted tumours showed the daily therapeutic dose level to vary between 1 5 and 3 mg /kg for the tumour-bearing rat, and 3 and 5 mg /kg for the tumour-bearing mouse   In testing the compound against a spectrum of rat and mouse tumours, treatment, usually with daily intraperitoneal injections, was begun when the tumours were from a pea to a bean in size   A prolonged treatment not involving considerable loss of weight was aimed at   After treatment varying in time with the strain of tumour, the animals were killed, the tumours removed and measured, and the percentage growth–inhibition was determined on the basis of the following formula

$$\text{Inhibition \%} = \frac{\text{Average weight of tumours in controls} - \text{Average weight of tumours in experimentals} \times 100}{\text{Average weight of tumours in controls}}$$

In some experiments survival time was used as a measure of benefit from the drug   In testing DBM against tumours in ascitic form, treatment was begun either one or two days after inoculation of the ascites, or on the first signs of ascites formation in the inoculated animal, and the degree of the inhibitory effect was graded according to the difference in body weight between the treated and the control animals

The drug was subjected to detailed examinations for its effect on the blood picture, the bone marrow, and the parenchymatous organs

In most cases the effectiveness of DBM was compared with that of Degranol (BCM)

RESULTS

Effect of DBM (R_{13}) on transplantable tumours

1 The growth-inhibiting action on rat tumours was studied in a set of experiments using Guérin carcinoma, Benevolenskaya sarcoma, and a subcutaneous form of Yoshida sarcoma   In agreement with Druckrey and co-workers (1958), we found the latter was just as useful as Yoshida ascites sarcoma in testing chemotherapeutic agents   rats inoculated with the tumour in ascitic form survived

for an average 8 to 10 days, with only 1 to 3 ml of ascitic fluid forming , subcutaneously inoculated rats survived for 12 to 18 days

The growth-inhibiting effect of DBM on the rat tumours involved in our experiments is illustrated in Table I and Fig 1 and 2

Using Guérin tumour, DBM was tested for its effect upon survival time in 39 Wistar strain rats treated in three groups with an intraperitoneal daily dose of 0 4, 1 0, and 2 0 mg /kg of body weight, respectively , another 39 rats of the same strain were left untreated to serve as controls , an additional 24 rats bearing Guérin tumours were given from 4 to 12 mg daily doses of Degranol per kg of body weight   Treatment was continued until the death of the last experimental animal   In relation to the untreated controls, DBM was found to have prolonged the survival time by from 8 to 15, and BCM by from 2 to 7 days

2   For its effect on transplantable solid mouse tumours the compound was studied on subcutaneously inoculated Ehrlich carcinoma and S-37 and S-180 (Crocker) sarcoma   The results obtained are summarized in Table II

3   For its growth-inhibiting action in mouse tumours in ascitic form, DBM was tested in Ehrlich carcinoma and Amytal sarcoma   The latter originates from this Institute (Juhász, Baló and Kendrey, 1955) , it is transplanted in every 10 to 12 days, during which time 8 to 10 ml of ascitic fluid develop in each animal , the mice generally survive transplantation by from 14 to 18 days   To every ml of ascitic fluid drawn from them, 0 25 mg of DBM and Degranol, respectively, were added dissolved in 1 ml saline under sterile conditions and the mixture was kept in the thermostat at 37° C for 45 minutes   Two groups of 10 white mice each were then inoculated with an equal amount of one and the other of these mixtures, respectively, while in a third group of 10 controls each animal was inoculated with a corresponding amount of a mixture of ascitic fluid and sterile physiological NaCl incubated likewise at 37° C for 45 minutes   All the animals were kept under observation for 11 days with a view to finding out to what extent under the identical conditions equal concentrations of the two drugs were capable of depressing takes and inhibiting tumour growth   The results of daily checks on the changes of body weight showed that while the average over-all gain was 7 10 g in the control group, it amounted to 3 23 g in the group treated with DBM, and to 5 74 g in that treated with Degranol , in other words, an inhibitory effect of 44 per cent was observed upon the action of DBM, and of 19 2 per cent upon that of Degranol

Using Amytal ascites sarcoma, another experiment was carried out to determine the influence of DBM and Degranol, respectively, on the survival time of mice   Fig 3 shows that while seven consecutive daily doses of 4 85 mg of DBM per kg of body weight resulted in prolongation of survival time, no appreciable prolongation followed the administration of the same number of 21 mg doses of Degranol

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EXPLANATION OF PLATE

Fig 1 —Showing 92 per cent growth inhibition on Guérin carcinoma after 19 doses of 2 6 mg /kg of DBM (R_{13})

Fig 2 —Showing 93 per cent and 84 per cent growth inhibition on subcutaneously inoculated Yoshida sarcoma after 9 doses of 1 75 mg /kg of DBM (R_{13}) and 10 mg /kg of Degranol (BCM), respectively

Fig 5 —Showing inhibition of development of Ehrlich ascites carcinoma by 7 daily doses of 4 85 mg /kg of DBM (R_{13})
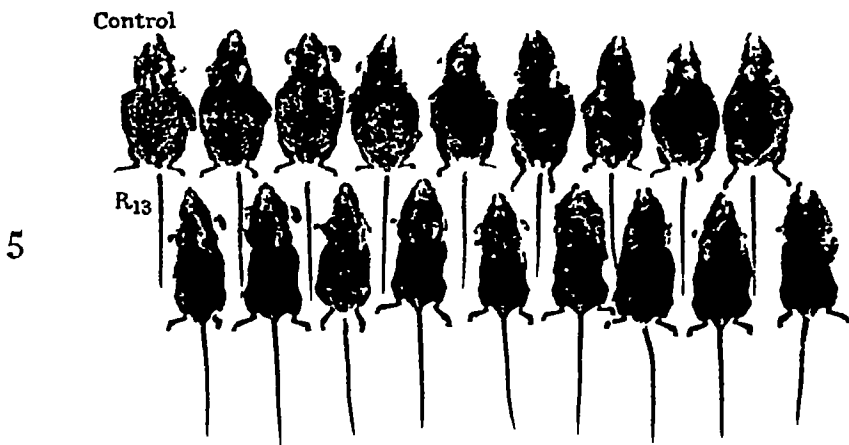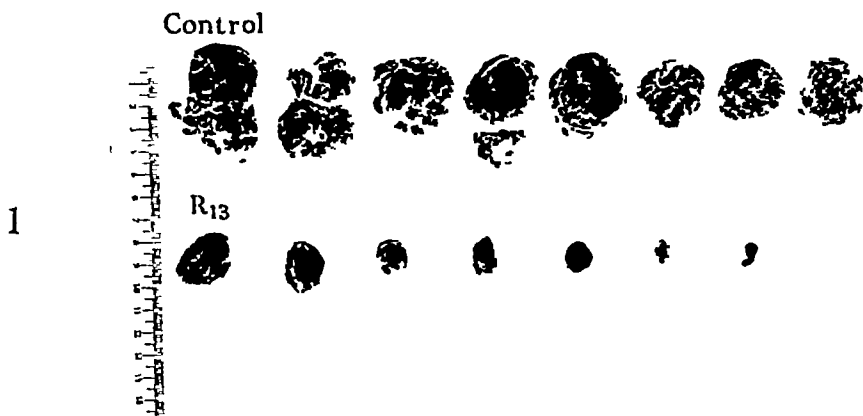
TABLE I—*Degree of Inhibition of the Growth of Certain Rat Tumours by DBM (R_{13}) and Degranol (BCM)*

Compounds tested	Tumours used	Number of rats		Daily dose (mg/kg)	Duration of treatment (in days)	Number of treatments	Average weight of tumours		Loss of weight (in %)	Inhibition of growth (in %)
		Treated	Control				Treated (g)	Control (g)		
DBM	Guérin rat carcinoma	15	15	2·6	49	24	7·80	70·00	—	89
DBM	,, ,, ,,	10	10	1·96	28	10	1·36	17·52	16	92
DBM	,, ,, ,,	11	11	12·00	17	12	2·10	5·82	—	64
Degranol	,, ,, ,,	11	11	1·85	17	12	2·70	5·82	—	34
DBM	Yoshida, rat sarcoma subcutaneous form	10	10	15·00	13	9	0·24	8·40	7	97
Degranol	Ditto	10	10	1·75	13	9	0·17	8·40	12	98
DBM	Ditto	8	8	10·00	14	8	1·22	16·77	0	93
Degranol		8	8	2·25	14	8	2·53	16·77	6	84
DBM	Benevolenskaya rat sarcoma	11	11		24	10	8·08	25·65	0	68
Degranol		11	11	12·75	24	10	8·86	25·65	3	66

TABLE II—*Degree of Inhibition of the Growth of Certain Mouse Tumours by DBM (R_{13}) and Degranol (BCM)*

Compounds tested	Tumours used	Number of mice		Daily dose (mg/kg)	Duration of treatment (in days)	Number of treatments	Average weight of tumours		Inhibition of growth (in %)
		Treated	Control				Treated (g)	Control (g)	
DBM	Ehrlich mouse carcinoma	8	8	3·00	12	8	1·33	2·18	34
Degranol		8	8	15·00	12	8	1·73	2·18	21
DBM	S 37 sarcoma	10	10	4·85	15	10	0·98	2·10	53
Degranol		10	10	14·00	15	10	1·71	2·10	28
DBM	S 180 (Crocker) sarcoma	12	12	4·85	12	9	1·55	3·61	57
Degranol		12	12	21·00	12	9	1·67	3·61	53

On the growth of Ehrlich ascites carcinoma, 11 consecutive daily doses of 6 75 mg of DBM per kg of body weight were found to have a marked inhibitory effect, while the same number of 14 mg doses of Degranol failed to inhibit essentially the growth of this tumour (Fig 4)
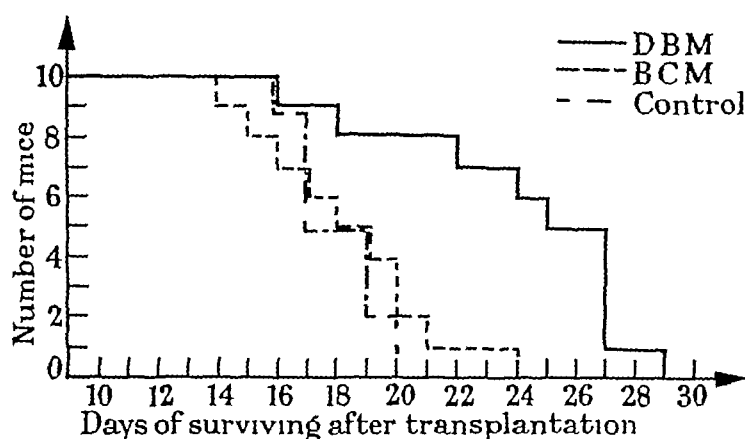
FIG 3 —Showing increase of survival time of mice inoculated with Amytal ascites sarcoma treated with DBM (R$_{13}$) or Degranol (BCM)
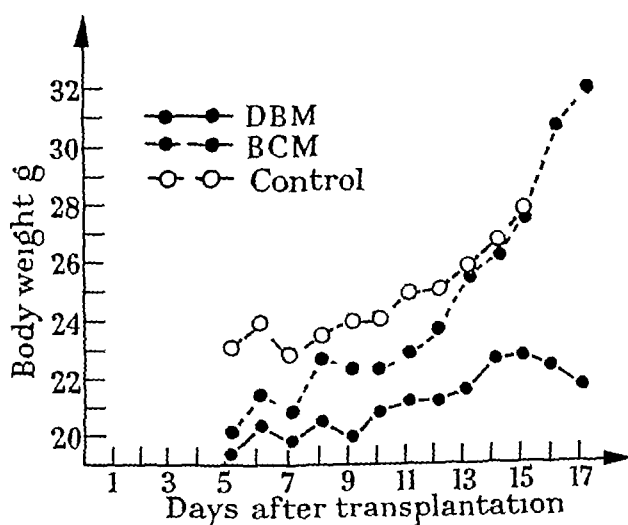
FIG 4 —Showing increase of weight of mice bearing Ehrlich ascites carcinoma following treatment with DBM (R$_{13}$) or Degranol (BCM)

In another experiment, in which animals bearing Ehrlich ascites tumours were treated during 9 days on each of seven occasions with 4 85 mg of DBM per kg of body weight, no ascites developed at all (Fig 5) Of Degranol, the same number of 21 mg doses was required to achieve the identical effect

Effect of DBM on blood picture, bone marrow, and parenchymatous organs

For its effect on peripheral blood DBM was studied in 16 rats of the Wistar strain, each weighing 150 g After their complete blood pictures had been prepared, some normal animals were injected intraperitoneally on one occasion with a simple, others with a threefold, yet others with a tenfold medium therapeutic dose (the

TUMOUR-INHIBITING ACTION OF DBM

sanustsimple *dosis therapeutica* being 1 75 mg /kg of body weight) Twenty-four hours later, there was no change in the number of erythrocytes at any dose levels, the simple therapeutic dose was followed by minimal lymphopenia , the threefold dose gave rise to significant lymphopenia and monocytopenia and relative leukocytosis, though the white cell count remained normal The tenfold dose caused appreciable leukopenia, the lymphocytes disappeared almost completely from the blood, the plasma of the retained ones becoming vacuolated and, in addition, a marked decrease in the number of monocytes and a relative leukocytosis were observed (Table III)

TABLE III —*Effect of a Single Intraperitoneal Dose of DBM Upon the Blood Cells*

Dose (mg /kg)		Number of erythrocytes (10⁶/c mm)	Number of leukocytes (10³/c mm)	Poly	Ly	Mo	Eo
1 75	Before treatment	6 4	8 2	42	55	2	1
	After treatment	7 8	9 2	43	53	4	0
5 25	Before treatment	8 5	10 7	36	59	3	2
	After treatment	7 9	9 1	62	37	0	1
17 5	Before treatment	8 5	9 2	25	72	1	2
	After treatment	8 4	6 6	92	6	0	2

In animals given the medium therapeutic dose for 15 consecutive days, there was no change in the red cell count, but the white cells fell to 5000 per c mm from the normal of about 9000 per c mm generally counted in the control animals , in the number of lymphocytes a substantial decrease was observed , relative leukocytosis presented itself, associated with eosinophilia (Table IV)

TABLE IV —*Effect of Serial Intraperitoneal Treatment (15 Times) with DBM Upon the Blood Cells of Rats*

Daily dose (mg /kg)		Erythrocytes (10⁶/c mm)	Leukocytes (10³/c mm)	Poly	Ly	Mo	Eo
1 75	Before treatment	8 2	9 0	34	62	2	2
	After treatment	8 3	5 0	45	46	3	6

For its action on bone marrow the compound was tested in normal intact Wistar strain rats which, in 45 days, had been treated on 34 occasions with 1 75 mg of DBM per kg of body weight by the intraperitoneal route This treatment was followed by a slight decrease in the number of myeloid elements In some individual cases the haemopoietic colonies were seen to have ceded their place to fatty bone marrow The blood pictures of these animals revealed marked lymphopenia, relative leukocytosis and moderate eosinophilia No pathological changes were encountered in the myocardium, the lung, the kidney, and the adrenal gland, but the spleen was found to have decreased in size, and the lymph follicles were less than normal in number Slight pulpar fibrosis was seen In the liver parenchymatous degeneration was observed, but no fatty degeneration or cellular necrosis

DISCUSSION

On the evidence of the results of our toxicity tests, the efficient but not toxic dose of DBM varies between 1 5 and 3 0 mg per kg of body weight for the tumour-

bearing rat, and between 3 0 and 5 0 mg for the tumour-bearing mouse   At these dose levels, the animals will stand treatment for 10 to 12 consecutive days without losing more than 6 to 12 per cent in weight   The chemotherapeutic index for DBM being

$$\frac{\text{MTD}}{\text{MED}} = 25,$$

it comes to fall between that for Degranol (50) and nitrogen mustard (10)

From our experimental findings the conviction accrues that DBM is a drug, which exerts a marked inhibitory effect on tumour cells   Undoubtedly, it was the most effective against Yoshida sarcoma, a tumour responding as a rule fairly well to efficient chemotherapeutic agents   Equally remarkable was the effect it appeared to have on Guérin cancer, the more so as malignant animal tumours (Guérin and Ehrlich tumours) are conspicuous for the difficulty in influencing their growth by means of therapeutic drugs   Not a single complete regression was observed in animals inoculated with these tumours, unlike in cases of sub-cutaneously implanted Yoshida sarcoma   Histologically, the small tumours found in a few of our animals inoculated with Yoshida sarcoma and treated with DBM could no longer be regarded as real tumours

In animals bearing Guérin or Ehrlich carcinoma, we on a few occasions suc-ceeded in maintaining for some considerable period of time a state in which there was no perceptible tumour growth, and even several remissions took place   If in such a state treatment was continued, the animals died after a time, displaying toxic symptoms , if it was discontinued, the tumour started to grow again but could not be depressed once more, not even by means of larger doses

With the histological changes in tumours following treatment with DBM, we propose to deal elsewhere

SUMMARY

Twenty-three compounds prepared by linking nitrogen mustard with various sugars were tested on various transplantable tumours for their capacity to inhibit growth   The one designated as DBM (R_{13}), the structural formula of which may be represented as 1,6-di-(2-bromoethylamino)-1,6-dideoxy-D-mannitol dihydro-bromide, was found to have a marked inhibitory effect on Guérin rat carcinoma, subcutaneous form of Yoshida rat sarcoma, Benevolenskaya rat sarcoma, the subcutaneous and ascitic forms of Ehrlich mouse carcinoma, Crocker and S-37 mouse sarcoma, and Amytal ascites sarcoma in the mouse   The drug is much less toxic than nitrogen mustard and depresses tumour growth without causing considerable loss of weight or toxic side effects   It is not without effect on peri-pheral blood and the lymphatic organs   Following treatment with therapeutic doses for some longer period of time, there is a reduction first in the lymphocyte and then in the leukocyte count, the spleen diminishes in size, the lymph follicles in it grow less in number, and fibrosis of the pulp is seen   Animal experiments have shown this agent to be more effective than Degranol

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AN OSTEOGENIC SARCOMA IN THE FOWL

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SPONTANEOUS primary bone tumours are so rare in the fowl that the occurrence of an osteogenic sarcoma is of sufficient intrinsic interest to warrant report, particularly since Feldman and Olson (1952) state that the rarity of these neoplasms has precluded adequate study

Osteomata have been observed in the fowl by Fujinami (1930) and Heim (1931) Kaupp (1933) described a tumour which he termed a sarco-chondro-osteoma Avian osteoclastomata have been reported by Baker (1928) and Campbell (1947) Tytler (1913) described in detail a transplantable osteo-chondro-sarcoma in fowls Eber and Malke (1932) mentioned the occurrence of an osteosarcoma among their large collection of avian neoplasms Reis and Nobrega (undated) state that in birds they have seen five osteogenic sarcomata, three of which presented the appearance of osteo-chondro-fibromata Neither Eber and Malke nor Reis and Nobrega described these tumours in any detail

This communication describes the finding of a malignant osteogenic sarcoma, which had arisen spontaneously at the proximal extremity of the right tibia, and had metastasised to the right kidney and to both lungs

Case history

A Brown Leghorn female (A 3466) was found to be lame when over seven years old She was one of a group of normal hens kept at the Poultry Research Centre under ordinary semi-intensive conditions as controls to a long-term environmental experiment, briefly reported on by Greenwood (1958) and Wilson (1958) A hard swelling had developed in the region of the right femoro-tibial articulation Movement of this joint was somewhat restricted but the degree of lameness was mild On palpation the swelling was found to be hard, apparently painless and firmly adhering to the bone A radiograph (Fig 1) showed a tumour involving the proximal extremity of the tibia , it affected neither the femur nor, apparently, the joint cavity The tumour was considerably calcified and the " explosive " pattern on the X-ray was very suggestive of that seen in human osteosarcomata The plasma alkaline phosphatase activity of this bird was 360 units (mg of 4-nitrophenol liberated by 100 ml: of plasma at pH 10, during 60 minutes at 37° C)

Post mortem findings

A large tumour, about 3 cm in diameter and of very firm consistency was situated near the proximal extremity of the tibia The femoro-tibial joint itself and the distal extremity of the femur were unaffected No other bones of the body were found to be involved Numerous very firm nodular metastases of up to 5 mm in diameter were scattered throughout the substance of the right

kidney   Both lungs carried similar tumours of varying size   The other organs,
including the left kidney, showed no macroscopic abnormality

Transmission experiment

No tumours resulted from injections of saline tumour suspension in day-old
chicks   Similar material grew on the chorio-allantoic membrane of 3 out of 6
inoculated 10-day chick embryos, but further passage failed

Histo-pathology

Material from the primary growth on the tibia was fixed in 10 per cent formal-
saline and decalcified   Blocks of other tissues and organs were fixed in Susa,
embedded in paraffin and sectioned without previous decalcification

The tibial tumour showed a structure typical of osteogenic sarcomata, with
large areas of osteoid and calcified bone (Fig 2) and, particularly at the periphery,
areas of more or less undifferentiated spindle-shaped osteoblasts (Fig 3)   The
osteoid and calcified bone was arranged in irregular trabeculae which were
bordered by chains or sometimes large groups of pleomorphic, somewhat spindle-
shaped osteoblasts, which frequently bore numerous processes (Fig 4)   In
section the free " edge " of the tumour showed sheets of closely knit undifferen-
tiated cells, exhibiting marked infiltrative growth into the surrounding muscle
tissue (Fig 5)   Although multinucleated giant cells may have been present
they were by no means obvious, even in the calcified portions of the neoplasm
In the undifferentiated peripheral portion mitotic figures, although present, were
not numerous , the nucleoli of the osteoblasts in these areas were, however,
large and very prominent (Fig 3)   The tumour was well vascularised and there
was no evidence of necrosis

The histological appearance of the metastases was essentially similar to that
of the primary tumour   In the lungs, as well as in the kidney, these secondary
growths appeared to be fairly well circumscribed and more or less spherical ,
they were not encapsulated and, in fact, manifested apparently rapid infiltrative
growth into the tissues of the affected organs

In the centre of these metastases, well differentiated and partially calcified
osteoid was present, particularly in the lungs   This was irregularly interspersed
by groups of readily recognisable osteoblasts   The peripheral infiltrating zone
had a much more anaplastic character, the tumour cells exhibiting great
pleomorphism and numerous mitotic figures (Fig 6)

The apparently rapid infiltrative growth of the metastatic tumour cells in
the kidney is illustrated in Fig 6, where atrophic, but in no way degenerate,
renal tubules and glomeruli were seen embedded in tumour tissue   In the lungs
the appearance was essentially similar in that the alveoli tended to become
filled with tumour cells, and in the peripheral portion at any rate, they retained a
suggestion of lobulated structure   Areas of necrosis were not observed in the
metastatic growths

DISCUSSION

There can be little doubt that this tumour is an osteogenic sarcoma   Its
histological and radiological characteristics are virtually identical with those of
human osteosarcomata   According to the classification of Coventry and Dahlin

(1957) the tumour resembles a human osteoblastic osteogenic sarcoma on the basis of the predominance of more or less anaplastic osteoblasts and the presence of a variable but definite osteoid component   Calcification was seen not only in the primary tumour but also to some extent in the metastases   According to Dahlin (1957) multinucleated giant cells are rarely seen in osteogenic sarcomata, in the present case such cells were not observed

It is unfortunately impossible to compare the histology of the present tumour with that of other osteosarcomata occurring in fowls   The only other cases which have been found in the literature were reported by Eber and Malke (1932) and Reis and Nobrega (undated), neither of whom give any histological details

Authorities agree that the most common sites for osteogenic sarcomata in man are the distal extremity of the femur and the proximal end of the tibia (Christensen, 1925 , Geschickter and Copeland, 1949 , Dahlin, 1957 , Amromin, 1959)   While in the avian case of Eber and Malke the primary tumour was situated on the sternum, in the present case it was found to be at the proximal extremity of the right tibia   Reis and Nobrega make no mention of the location of their tumours

Two-thirds of the human osteogenic sarcomata occur at an age of between 10 and 30 years , very young children and old people are seldom affected (Willis, 1953)   If a true comparison were at all possible, the present case would be found to fall very much outside this range , at the time when the tumour was diagnosed the hen was over seven years old, an advanced age for a domestic fowl   It is possible that one reason why this counterpart of the most common primary malignant human bone tumour is so rarely observed in fowls, is that they are not kept commercially beyond a maximum age of about 3–4 years

The plasma alkaline phosphatase activity in this present case was 360 units , the range for non-laying, non-moulting, normal hens lies between 25 and 120 units (Bell, 1960)   High alkaline phosphatase activity is also characteristic of human osteogenic sarcomata (Willis, 1953)   The alkaline phosphatase of the plasma is believed to originate largely if not entirely, in the osteoblasts , the enzymic activity of the plasma may therefore be considered to reflect the amount of osteoblastic activity in the bone   In birds suffering from " cage layer fatigue ", which involves a bone dystrophy, high values for plasma alkaline phosphatase (300–600 units) have been invariably found (Bell, Siller and Campbell, 1959)

EXPLANATION OF PLATES

FIG 1 —Radiograph of the right leg, showing a large calcified tumour at the proximal end of the tibia   The femur and the femoro tibial joint were not affected   Note the partially calcified tendons

FIG 2 —Section of the primary tumour shown in Fig 1   Note the high degree of differentia tion with the formation of osteoid and calcified bone   There are large masses of mature osteoblasts, but no osteoclasts   H & E ×95

FIG 3 —Illustrates the more anaplastic character of the osteoblasts at the periphery of the primary tumour   H & E   ×385

FIG 4 —Calcified and non calcified osteoid of the tibial neoplasm, showing various degrees of maturity in the osteoblasts , some bear very pronounced processes   H & E   ×385

FIG 5 —Infiltrative growth of the primary tumour   Note the masses of anaplastic osteoblasts invading the neighbouring muscle tissue   H & E   ×95

FIG 6 —Metastasis in the right kidney, showing atrophied tubules and glomerulus and also mitotic figures   H & E   ×385
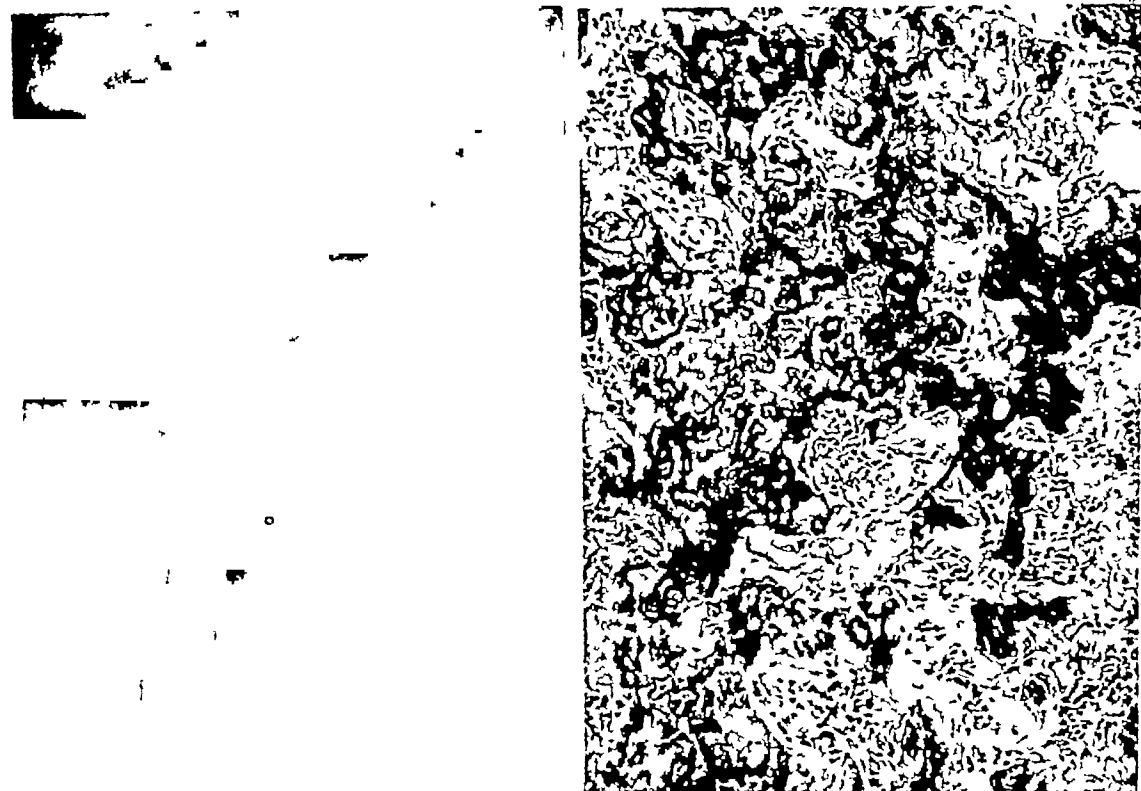
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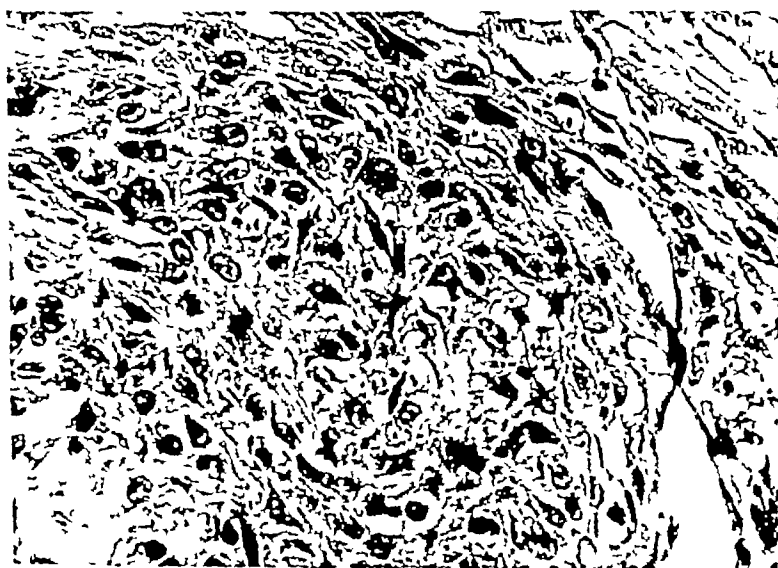
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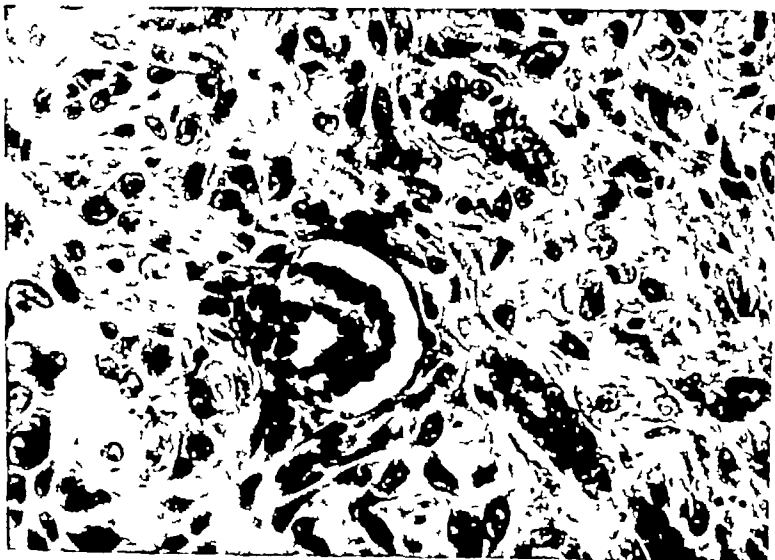
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In view of the fact that some bone tumours in the fowl, such as the osteoma of Fujinami (1930), the osteo-chondro-sarcoma of Tytler (1913) and the osteoid sarcoma of Pikowski and Doljanski (1946), are transplantable, it is noteworthy that the transplantation experiments failed in the present case

Metastases to the lung occur very frequently in man Willis (1953) assesses the incidence of secondary growths in the lungs as high as 75 per cent Spread is generally by the haematogenous route and lymphatic dissemination is rare In the present case both lungs carried metastases A very interesting phenomenon is the confinement of renal metastases to that organ situated isolaterally to the affected leg Birds possess a renal portal circulation (Spanner, 1924–25), whereby venous blood from the leg is filtered through the capillary bed of the isolateral kidney The fact that heavy metastases were found in the right kidney, and none in the left, supports the conclusive work of Sperber (1948) on the existence of a functional renal portal system This would provide an immediate haematogenous pathway between the primary tumour and the capillary bed in the kidney Despite Sperber's experimental results and his carefully summarised evidence for and against the existence of a functional renal-portal system there are some (e g Barth, 1949) who still question its existence in the adult fowl

SUMMARY

A case of spontaneous osteogenic sarcoma is described in a seven-year-old female fowl The primary tumour developed on the proximal extremity of the right tibia and there were metastases in the right kidney and both lungs The radiological and histological examination of this neoplasm showed the characteristic picture of human osteogenic sarcomata A high plasma alkaline phosphatase activity was observed in this fowl Transmission experiments were negative

The occurrence of the isolateral renal metastases is interpreted as additional evidence for the existence in adult fowls of a functional renal portal system

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ON A HORMONE-DEPENDENT SPONTANEOUS, MALIGNANT ASCITES TUMOUR IN RATS

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In recent years much interest has been focused on ascites tumours They were originally obtained from primary tumours in rats and mice that had been treated in some way or other The ascites tumours were then transplanted in series through a large number of animals One of the types of tumours developing in rats was the Yoshida sarcoma Cells from this tumour proliferate in the abdominal cavity and sometimes infiltrate surrounding tissues with formation of solid, massive growth, which soon kills the host Thick ascites, a suspension of tumour cells, forms in the peritoneal cavity This material can be injected into the abdominal cavity of normal rats and the tumour thus carried in series Other similar rat sarcomas are Takeda-, MTK- and Hirosaki sarcoma (Yoshida, 1953)

A search of the literature failed to reveal any report on the spontaneous occurrence of primary ascites tumours in untreated rats

This paper is concerned with a spontaneous ascites sarcoma that was found to be fairly common in a closed rat colony The tumour was successfully transplanted by intraperitoneal, subcutaneous and intravenous injection It was made the subject of chromosomal studies and proved to be dependent on sex hormones

In the beginning the tumour appeared to be more common in males than in females In an attempt to reveal any relationship between sex hormones and the development of the tumour, groups of intact male and female rats, castrated males, and spayed females were compared One group of spayed rats received oestrogen In addition, the frequency of the tumour was studied in parabiotic rats, of which one partner was an intact and the other a spayed female Gonadotrophins from the spayed female pass over to the intact female and stimulate its ovaries (Zeckwer, 1946) In the intact female partner in parabiosis the oestrogen production therefore is fairly constant and relatively high The oestrogens are not transferred to the spayed partner

MATERIAL AND METHODS

The observations were made in animals of one and the same closed colony that has been maintained, without brother-sister mating, at the Department of Obstetrics and Gynaecology in Lund All the animals have been brought up in cages of approximately equal size and on the same diet consisting of grain, milk and kitchen refuse

Some of the animals—males and females—were castrated or spayed at 3 weeks of age, others were left intact Some of the spayed and intact female rats of

the same litter were joined in parabiosis, coelio-anastomosis, at 4 weeks of age
Some of the spayed females received oestrogens in oil solution (0 25 ml 1 m
= 1 25 mg oestradiolbenzoate/week) for 11 months

The experimental material is given in Table I

TABLE I —*Number of Experimental Animals Above* 9 *Months of Age*

(The first tumour was observed at this age)

Intact males	45
Castrated males	50
Intact females	134
Spayed females	102
,, ,, treated with oestrogen	48
,, ,, in parabiosis with intact animals	24
Intact females in parabiosis with spayed	24
Total	427

Most of the animals were allowed to live until they died spontaneously   Some
were killed to secure fresh material for histological examination   All the animals
were examined *post mortem*

RESULTS

The tumour was never observed in intact or spayed females before the animals
were 9 months of age   In males they did not appear until still later   The
oldest animal in which a tumour of this type developed was a spayed female,
20 months old   The frequency and age distribution of the animals killed by
the tumour during the period of observation are given in Fig 1

Statistical analysis showed that the material permitted no definite conclusions
regarding the frequency of the tumour in the different groups of animals until
the animals were more than 12 months old   After this age limit the frequency
of tumours was higher in spayed females compared with intact females and
spayed rats treated with oestrogen   The difference between the frequency
expected and that observed was 4 times the mean error

No difference in frequency of the tumour was found between spayed females
and spayed females that had lived in parabiosis with intact females   Parabiosis
between spayed females and intact females produced no statistically significant
inhibition of tumours in the intact females

Neither was any difference found between the frequency of tumours in males
and females or between intact and castrated males

As a rule, the animals died within 2–3 weeks after the appearance of demon-
strable symptoms   They lost weight rapidly and became severely anaemic
The abdomen swelled severely with ascites and tumour (Fig 2)   At autopsy
the peritoneal cavity was filled with cloudy, usually slightly blood-stained fluid
Sometimes it was also milky or almost water-clear   The ascitic fluid contained
a large number of tumour cells loosely dispersed or in small clumps   The nuclei
of the cells varied in size and shape   The appearance of the tumour cells is so
characteristic that the cells are readily distinguished from blood cells (Fig 3)
Chromosomal analysis was performed at the Cancer Chromosome Laboratory
in Lund (Hansen-Melander and Melander, unpublished)   Excellent material for
chromosomal studies is also obtained from the soft solid tumours

The solid tumours, usually very widespread, were soft and richly vascularised The tumour showed a distinct tendency to haemorrhage and necrosis, sometimes with secondary infection   The tumour tissue grew in the omentum or on the peritoneum and in large confluent infiltrates in the ileocolic mesentery or retroperitoneally (Fig 4)   The cut surface of the tumour was solid and white or slightly pink   The mediastinum often showed metastases, usually adherent to and infiltrating the thymus   Then the pleural cavities contained fluid of the same type as the ascites in the abdomen   Metastatic growth to the lymph nodes

45	45	45	45	44	43	42	42	42	40	39	39	Living rats			
				■									I		♂
50	50	49	49	49	48	46	45	43	43	43	41	Living rats			
					■	■	■ ■						II	Castrated	♂
134	129	128 ■ ■ ■	125 ■	102	101	100	100	95	94	57	57	Living rats			
■	■	■											III		♀
102	101	100	98 ■	81 ■	77 ■	75 ■	73 ■	71	71	49	48 ■	Living rats			
■	■	■											IV	Spayed	♀
48	48	48	48	48	47	47	47	46	45	45	41	Living rats			
				■+H									V	Spayed (Oestrogen-injections)	♀
24	23	20	20	19	17	14	13	12	11	3	3	Living rats			
■			■	■	■								VI	Spayed (Parabiosis with VII)	♀
24	23	20	20	19	17	14	13	12	11	3	3	Living rats			
	■												VII	(Parabiosis with VI)	♀

9   12   15   18   21
Months of age

FIG 1 —Each filled square indicates one rat dying from spontaneous ascites tumour   Only one animal, in Group V, had a co existent tumour elsewhere (in hypophysis)   The numerals designate survivals in various age classes

in the groins, axillae and neck was observed   On the other hand, the liver, lungs, kidneys and spleen appeared not to be involved, not even microscopically   The largest tumours were situated in the ileocaecal mesentery, which appeared to be the original site of the tumour, possibly in the lymphatics which there run parallel to the ascending colon and extend cranially along the vasa mesenterica superior The outer layers of the wall of the colon were seldom infiltrated, and the intestinal mucosa was intact

The tumours, both the large retroperitoneal infiltrates and the various metastases showed the same microscopic picture (Fig 5) with diffuse growth of anaplastic tumour cells cellular and nuclear polymorphism   The cytoplasm was relatively sparse and acidophilic   Cells with large, lobated nuclei were common The nuclei often lay eccentrically in the cells   Mitoses were extremely common The stroma was scanty and consisted of a few fine fibrils or trabeculae, rich in

small blood vessels   Haemorrhages, necroses and infiltration with lymphocytes
and leucocytes were common   The tumour may possibly be regarded as a poly-
morpho-cellular reticulum-cell sarcoma

The rats used as hosts in the transplantation experiments belonged to the
same colony and were, as a rule, 1–2 months old   After intraperitoneal inocula-
tion of 0 5 c c ascites or a small piece of homogenised tumour suspended in
saline the ascites sarcoma grew rapidly in some of the new hosts   After a few
passages successful inoculation usually killed the animals within two weeks
During the last few days before the animals died large amounts of ascitic fluid
accumulated in the abdomen, which was markedly distended   In addition, solid
growths always occurred in one or more of the sites where the primary tumours
had been found   Inoculation was followed by tumour growth in males and
females, gonadectomised or intact, and independently of the fact whether they
had received ascitic fluid or homogenised solid tumour

For some unknown reason in some of the animals the primary tumour failed
to take   After rather many successful passages the tumour sometimes suddenly
failed to take   This might have been due at least in part to genetical hetero-
geneity of the strain and chromosomal changes in the tumour during its passages
Of 15 primary tumours takes were obtained with 7   One tumour was carried
through 18 generations   After subcutaneous inoculation of ascites solid tumours
developed at the site of inoculation   After intravenous injection (into the tail
vein) of ascites or homogenised solid tumour an extensive tumour growth was
observed in the same sites as after intraperitoneal inoculation

DISCUSSION

The growth of ascites cancer cells in heterologous hosts was found by Ahlstrom
and Ising (1955, 1956) to vary with sex   On transplantation of mouse ascites
carcinoma to hamsters growth was more abundant and the yield of tumour cells
larger in females than in males

The tumour described here, probably a reticulum-cell sarcoma, growing also
in ascitic form, and probably of high degree of malignancy (developed early in
life of the animals and killed them rapidly) was found to be more common after
oophorectomy and to be inhibited by administration of oestradiol   Tumours in
the lymphatic tissue (lymphosarcoma) in mice, on the other hand, occur more
frequently in animals treated with oestrogens (Shimkin, 1957)

SUMMARY

A spontaneous ascites tumour probably a reticulum-cell sarcoma, that devel-
oped in a closed rat colony is described   All of the animals with such a tumour
died between 9 and 20 months of age

EXPLANATION OF PLATE

Fig 2 —Rat with spontaneous malignant abdominal tumour producing ascites   One week
    before death
Fig 3 —Tumour cells in ascitic fluid. Papanicolaou   × 600
Fig 4 —Extensive growth of partly haemorrhagic tumour in mesentery and omentum
    (compare Fig 2)
Fig 5 —Tumour growth in omentum   6 μ section   Haematoxylin and eosin   × 600

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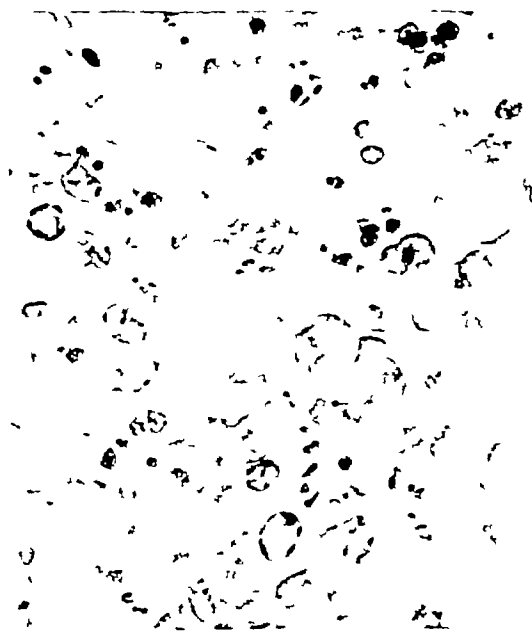
Development of the tumour is favoured by oophorectomy, and inhibited by administration of oestradiol Sometimes the tumour can be carried in series by intraperitoneal, subcutaneous or intravenous injection of ascitic fluid

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CHANGES IN THE OVARIES OF MICE TREATED WITH DIMETHYL-BENZANTHRACENE AND OBSERVATIONS ON THE SUBSEQUENT DEVELOPMENT OF TUMOURS IN OVARIES AND BREASTS

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It has previously been shown that fortnightly skin paintings of an oily solution of dimethylbenzanthracene (DMB) induce a high incidence of granulosa-celled tumours of the ovary, as well as of breast tumours, in mice of the IF strain and its first generation hybrids (Howell, Marchant and Orr, 1954 ; Marchant, 1957) The earliest ovarian tumour was encountered after 4 months of treatment

In a subsequent experiment ovaries from F_1 C57 Bl × IF mice, which had been treated with DMB for 3 months, were transplanted bilaterally to the ovarian capsules of untreated mice   In 14 out of 18 of these animals large ovarian tumours were found 15 months later, many of them being luteinised (Marchant, 1959a)   At the same time a preliminary account was given of the incidence of ovarian tumour nodules found in a series of DMB-treated mice killed at monthly intervals after beginning the treatment, for it was important to know whether or not incipient tumours were already present in the transplanted ovaries

The present report gives further details of this attempt to discover at what stage in DMB treatment tumour nodules first appeared in the ovaries   It describes the histological changes that preceded the appearance of granulosa-celled tumours, as well as those that occurred simultaneously in non-tumourous ovaries   It also gives observations on the luteinisation of established granulosa-celled tumours, which was not seen in mice of the IF strain or C57 Bl strain reported previously (Marchant, 1957)   Finally, it reports the incidence and histology of the palpable breast tumours which appeared after the 4th month of the experiment

MATERIALS AND METHOD

Eighty-five virgin female mice lacking the mammary tumour agent were used in this experiment   They were F_1 hybrids derived from C57 Bl mothers and IF fathers   They were housed 5 in a box and fed on rat cubes known as the Thompson diet

When the mice were between $2\frac{1}{2}$ and 4 months old, fortnightly skin paintings of 0 5 per cent DMB in olive oil were commenced   Up to 6 treatments were given, each mouse receiving about 0 2 ml (1 mg) distributed in 16 spots over dorsal and ventral surfaces at each painting

Ten mice were killed at monthly intervals at the beginning of the experiment After the 4th month breast tumours made their appearance, growing rapidly and necessitating the killing of some animals at irregular times   On account of this,

the results reported below include some mice which had to be killed within 2 weeks of the specified month

For a short while before death, vaginal smears were taken from nearly all the mice surviving 4 months or more, to determine whether the ovaries were secreting oestrogen or not

At autopsy the mice were examined for palpable breast tumours many of which were removed for histological examination   All ovaries were examined and removed   Those from mice up to and including the 5th month of treatment were serially sectioned   After that time representative sections from several different levels were mounted   Sections were stained with haematoxylin and eosin

The ovaries of 5 untreated mice aged 6 months were also examined and serially sectioned

In the early stages follicles and corpora lutea in the largest sections from each ovary were counted under low power to give a rough quantitative estimate of changes

<p style="text-align:center">RESULTS</p>

Ovaries

Untreated mice —The ovaries of the 5 untreated mice which were 6 months old, were about 3 × 4 mm in diameter, roughly kidney-shaped, coloured pale pink and studded with bright pink white or pale yellow spots

In histological sections   numerous follicles and corpora lutea were seen, bounded by a stroma of fibrous and vascular tissue (Fig 1)   The follicles occasionally contained 2 oocytes   They, together with the corpora lutea were situated towards the periphery   A few tiny clusters of lipochrome cells were seen   The largest sections from each of the 10 ovaries were examined in detail   They contained from 12 to 22 follicles with granulosa-cells (mean 15 8) and 13–27 fragments of corpora lutea (mean 19 4) per section

One month DMB treatment —After 1 month of DMB treatment (2 fortnightly paintings) the ovaries of the 10 mice examined appeared similar to those of untreated animals

Histologically, too   there was little difference from normal   The largest sections however, contained slightly fewer follicles and corpora lutea   The follicles numbered 5–19 (mean 12 7) and the corpora lutea 9–24 (mean 17 5)   In 4 of the animals a few of the corpora lutea appeared to be merging

Two months DMB treatment —After 2 months of DMB treatment the ovaries of the 10 mice killed appeared macroscopically normal   In histological sections a slight increase in numbers of merging corpora lutea was seen and the numbers of follicles counted in the largest sections of each ovary had fallen considerably   The follicles numbered 0–8 (mean 3) and the numbers of individual corpora lutea were 8–23 (mean 15 5)

Three months DMB treatment —After 3 months of DMB treatment (6 fortnightly paintings) only 1 mouse of the 10 killed had what appeared to be normal ovaries macroscopically   The other ovaries in the group were atrophied fairly smooth and yellowish

All 20 ovaries were very abnormal histologically (Fig 2)   In the largest sections no follicles were found   but examination of all the serial sections revealed

1 graafian follicle in 1 ovary of 4 different mice, and a 5th mouse had 3 graafian follicles in 1 ovary   Fusion of corpora lutea was much more marked in this group of animals and in 7 ovaries no distinct corpora were found   The ovaries of the mouse, which had appeared to be normal macroscopically, showed the least degree of fusion of corpora   In 1 mouse, in which no follicles were found and fusion of corpora lutea was well advanced, a nodule of tissue resembling granulosa-celled tumour was found, similar in size to a ripe follicle or corpus luteum   It is considered that this was the earliest certain tumour nodule found in this experiment   Four ovaries with fused corpora lutea contained tiny cysts

Four months from 1st DMB treatment —After 4 months of DMB treatment (6 fortnightly paintings) all the 10 mice killed had abnormal ovaries   In 3 mice the 2 ovaries were unequal in size, 1 ovary being about normal in size, the other slightly atrophied, yellowish and smooth   In the larger ovaries of these 3 mice small granulosa-celled tumours were found, the largest being nearly 2 mm diameter and making up about half the volume of the ovary   These small tumours were relatively undifferentiated or showed pseudofollicular differentiation   One was made up of 2 small nodules, 1 of which is illustrated in Fig 4 and 5   Vaginal smears of all 3 mice showed predominantly oestrus smears about the time of death

In the other 7 mice, both ovaries were slightly atrophied, yellowish and smooth   Histologically these ovaries showed a complete absence of follicles and oocytes from all sections   Corpora lutea were rarely distinct bodies and most of them were fused into masses of lutein tissue   From this time onwards considerable variation began to occur in the types of cells comprising the lutein tissue   Sometimes the cells were vacuolated or pigmented and in some cases the lutein tissue was converted in part to collagenous tissue, resembling human corpora albicantia   This condition has been referred to as " hyalinised " (Fekete, 1946 , Atkinson, Dickie and Fekete, 1954)   Vaginal smears showed predominantly dioestrus smears in 3 mice and anoestrus smears in the others   In one of the former, a tiny nodule suspected of being an incipient tumour was found

Five months from 1st DMB treatment —Fourteen mice were killed about 5 months from the 1st DMB treatment, 10 of which had palpable breast tumours   Macroscopically their ovaries were more variable than at 4 months   One had a dark red lump about 13 mm in diameter in 1 ovary, which proved to be a blood clot on histological examination

Another mouse had an enlarged ovary, about 5 mm diameter, divided into 2 separate lobes   One lobe was pink and this proved to be a granulosa-celled tumour which was slightly luteinised   The other lobe, like the contralateral ovary, was very pale yellow and knobbly   This mouse had predominantly dioestrus vaginal smears

Four mice had ovaries unequal in size, the larger in each case proving to contain a small granulosa-celled tumour   Three of these had oestrus smears and 1 had anoestrus smears

One mouse had smooth, pale pink ovaries, about normal in size, which were found to be invaded by lymphocytes   This mouse had a thymic tumour and generalised lymphomatosis, a condition found in about 20 per cent of the mice killed from 5 months onwards

The other 8 mice in this group had atrophied ovaries which were pale yellowish colour and smooth or knobbly   Like non-tumourous parts of tumourous ovaries,

on histological examination they showed diffuse luteinisation (Fig 3) with varying degrees of hyalinisation the knobbly ovaries being the most hyalinised   There was a complete lack of follicles   occasional small clumps of pigmented cells or scattered mast cells   Two of these mice had ovaries containing small granulosa-celled tumours and they had predominantly oestrus smears   Two others had suspected tumour nodules in them   1 having dioestrus and the other anoestrus smears (Fig 7 and 8)   The other 4 mice with atrophied ovaries had anoestrus smears

Six months from 1st DMB treatment —Eleven mice were killed about 6 months from the 1st DMB treatment   9 of them having palpable breast tumours

One of these mice had a cyst 5 mm in diameter in 1 ovary   It was filled with clear fluid and in its walls the ovarian tissue was embedded   This was found to be a luteinised tumour   The mouse had anoestrus smears

Another mouse had ovaries unequal in size, the larger containing a small granulosa-celled tumour   the vaginal smears being oestrus

A third mouse had ovaries about normal in size, pale yellow and studded with white spots   This appearance was due to very marked hyalinisation of corpora lutea   A small luteinising tumour was found in 1 ovary and the vaginal smears were predominantly oestrus

The other 8 mice had smooth, yellowish atrophied ovaries   A small granulosa-celled tumour was found in 1 of them and another had a suspected tumour and oestrus smears   The remainder had diffusely luteinised ovaries with, on the whole, a greater degree of hyalinisation then after 5 months and an increased number of mast cells present   They had anoestrus smears, with the exception of 1 mouse which had predominantly oestrus smears and whose ovaries were almost completely hyalinised

Seven months from 1st DMB treatment —Fourteen mice were killed about 7 months after the 1st DMB treatment, 13 of these having palpable breast tumours   One mouse had a purple lump about 9 mm diameter in 1 ovary, which proved to be a blood clot   The other ovary was atrophied and was found to be completely invaded by lymphocytes

Another mouse had in 1 ovary a cyst about 8 mm diameter filled with clear fluid and a luteinised tumour was embedded in its walls   It had anoestrus vaginal smears

Five mice had 1 enlarged ovary measuring between 4 and 8 mm diameter All of these contained granulosa-celled tumours and in 2 there were signs of luteinisation of the tumours   Four of the 5 mice had predominantly oestrus smears and the other had anoestrus smears

Five mice had unequal but un-enlarged ovaries, the smaller being atrophied The larger ovary of 1 of these proved to be a blood-filled cyst, but the contra-lateral atrophied ovary contained a small granulosa-celled tumour   This mouse had oestrus smears   Another had both ovaries very heavily invaded by lympho-cytes and its vaginal smears were oestrus   The bigger ovary may well have con-tained a tumour but it was impossible to be certain because of the lymphocytic infiltration   It is therefore scored as a suspected tumour   In the other 3 mice with unequal ovaries a small granulosa-celled tumour, was found in the larger ovary   In 1 case it was partly luteinised (Fig 9 and 10)   One of these mice also had a small tumour in the smaller ovary   This mouse had anoestrus smears and the other 2 had oestrus smears

The remaining 2 mice had atrophied ovaries and 1 contained a small cyst lined by ciliated epithelium   Both had anoestrus smears   All atrophied ovaries were diffusely luteinised and hyalinised to varying degrees (Fig 4)   Mast cells were frequently present, as were patches of large pigmented cells

Eight months from 1st DMB treatment —Six mice survived 8 months from the first DMB treatment, all developing breast tumours

A solid yellow tumour about 9 mm diameter was found in 1 mouse   It was a pseudofollicular granulosa-celled tumour, showing all stages of luteinisation (Fig 11 and 12)   Vaginal smears were oestrus

Another mouse had unequal ovaries, the larger being divided into a yellow and a dark red lobe, each about 3 mm diameter   The dark red lobe, contained a slightly luteinised granulosa-celled tumour, and blood-filled cyst   This mouse had anoestrus smears

A 3rd mouse had both ovaries slightly enlarged—about 4 to 5 mm diameter One was red and yellow and enclosed in a small cyst filled with clear fluid and the red part proved to be a blood-filled cyst   The other was yellow in colour and

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EXPLANATION OF PLATES

All figures show sections of ovaries of F_1 C57 Bl × IF mice treated fortnightly with dimethyl benzanthracene (DMB)   Haematoxylin and eosin stained

Fig 1 to 4 show non tumourous ovaries

Fig 1 —Ovary of untreated mouse aged 6 months   Follicles in various stages of development and corpora lutea are present   × 28

Fig 2 —Ovary of mouse aged $5\frac{1}{2}$ months which had received 3 months DMB treatment All follicles have gone, but degenerate remains of oocytes are still present   × 32

Fig 3 —Ovary of mouse aged 7 months which had begun DMB treatment 5 months pre viously   Few degenerate oocytes remain, corpora lutea are fused and traces of hyalinisa tion are present   × 28

Fig 4 —Ovary of mouse which had begun DMB treatment 7 months earlier   There is a marked degree of hyalinisation of lutein tissue   × 28

Fig 5 to 8 show lesions suspected of being early granulosa celled tumours

Fig 5 —Ovary of mouse which had begun DMB treatment 4 months earlier   Amongst eosino philic lutein tissue, a small nodule of more basophilic cells is seen   A larger separate nodule was found in this ovary   Vaginal smears were oestrus   × 60

Fig 6 —Same nodule as Fig 5 under greater magnification   Nuclei of many of the cells show prominent nucleoli and nuclear membrane, typical of larger tumours (see Fig 12) Granulosa cells of normal follicles have nuclei which stain intensely with haematoxylin and in which no structure can be seen   × 120

Fig 7 —Part of a diffusely luteinised ovary containing the smallest suspected tumour nodule found   Five months after DMB treatment began   Vaginal smears anoestrus   × 60

Fig 8 —Same nodule as Fig 8   The cells show a whorled arrangement with fibroblasts separating them and more basophilic cytoplasm than surrounding lutein tissue   This whorled arrangment is typical of some of the undoubted early tumours seen in these hybrid mice   × 120

Fig 9 to 12 show ovaries entirely made up of tumour and undergoing luteinisation

Fig 9 —Small ovarian tumour from a mouse killed 7 months after first DMB treatment   A portion of the tumour is still granulosa celled, but the greater part of it is considerably luteinised   Vaginal smears oestrus   × 28

Fig 10 —A luteinised area from the same tumour as Fig 9   Some of the luteinised cells are pigmented   × 120

Fig 11 —Part of a large ovarian tumour found 8 months after DMB treatment began   Cells of undifferentiated granulosa celled tumour in nodules are surrounded by areas of luteinised tumour cells   Vaginal smears oestrus   × 28

Fig 12 —Part of Fig 11 at higher magnification   Cells typical of undifferentiated granulosa celled tumour can be seen adjacent to heavily luteinised tumour cells   × 120

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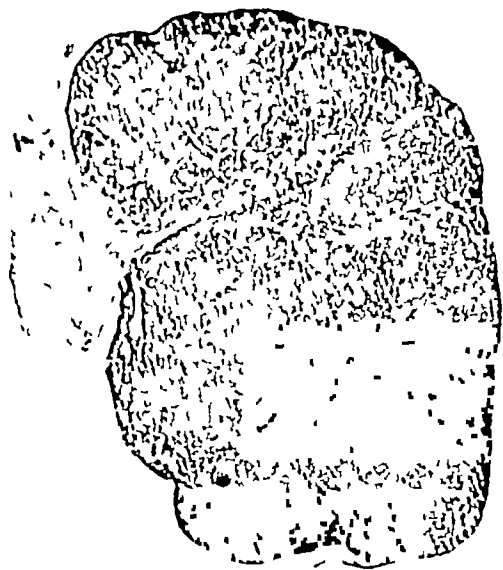
diffusely luteinised, with a small pseudofollicular granulosa-celled tumour in it
Vaginal smears were predominantly oestrus

The other 3 mice had atrophied ovaries   These were all diffusely luteinised,
with pigmented cells and prominent germinal epithelium   In 1 case, the latter
was invaginated and there was slight invasion of the ovaries by lymphocytes
In another very small ovary, there were clusters of anovular follicules and an
invaginating germinal epithelium   Both of these mice had predominantly
dioestrus smears   The 3rd had a small granulosa-celled tumour in 1 ovary and a
suspected tumour nodule in the other   Its smears were anoestrus
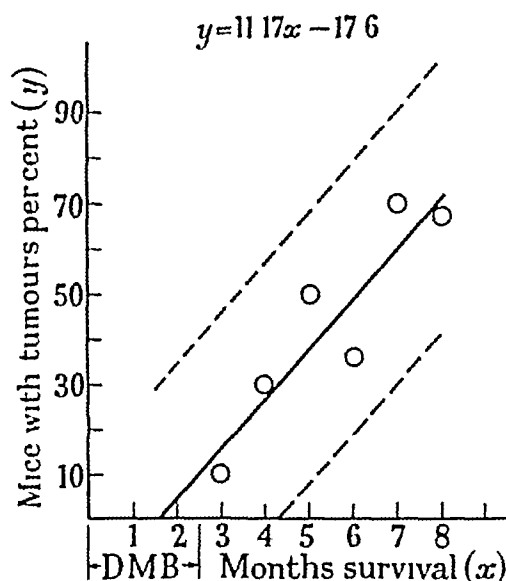
FIG 13 —Increase of detectable neoplastic changes in ovaries of DMB treated mice with time

Ovarian tumour incidence and survival —The incidences of granulosa-celled
tumours which occurred in the different groups of mice is given in Table I   The
incidence has been plotted against survival time in Fig 13 and it can be seen that

TABLE I —*Incidence of Granulosa-celled Ovarian Tumours
by Size per Month since 1st DMB Treatment*

Months since 1st DMB	Total mice	Without tumours	Suspected tumour nodules	Histo-logically detected tumours	Tumours in unequal ovaries	Tumours in enlarged ovaries	Mice with ovarian tumours (per cent)
0	5	5	0	0	0	0	0 (0)
1	10	10	0	0	0	0	0 (0)
2	10	10	0	0	0	0	0 (0)
3	10	9	0	1	0	0	1 (10)
4	10	6	1	0	3	0	3 (30)
5	14	5	2	2	4	1	7 (50)
6	11	6	1	2	2	0	4 (36)
7	14	3	1	2*	4*	5	10 (70)
8	6	2	1*	1*	0	3	4 (67)
Total	90						

* Bilateral tumours
† Excluding suspected tumour nodules

there is a roughly linear increase of incidence with time   The regression line and its 95 per cent confidence limits are shown on the figure

Table II summarises month by month the main histological structures found in the ovaries of the C57 Bl × IF hybrid mice treated with DMB

TABLE II —*Structures Present in Ovaries of F_1 Hybrid C_{57} Bl × IF Mice Treated with DMB*

| Months DMB | Follicles | Corpora lutea | | | Ovarian tumours | | |
		Separate	Fused	Hyaline	Suspected	Granulosa celled	Partly luteinised
0	+ +	+ +	—	—	—	—	—
1	+ +	+ +	±	—	—	—	—
2	+	+	+	—	±	—	—
3	±	+	+	—	—	±	—
4	—	±	+ +	+	±	+	—
5	—	—	+ +	+	±	+ +	—
6	—	—	+ +	+	±	+	±
7	—	—	+	+	±	+	+
8	—	—	+	—	±	+	+

+ + Abundant   + Present   ± Sometimes present, sometimes absent   — Absent

Breast tumours

Breast tumours occurred after the 4th month from the beginning of DMB treatment   After the 5th month, many of the mice had more than 1 palpable tumour when killed   Table III gives the numbers of tumours developed per mouse and the incidence of mice with palpable breast tumours

TABLE III —*Incidence of Mice with Palpable Breast Tumours per Month Since First DMB Treatment*

| Months DMB | Total mice | Number of palpable breast tumours per mouse | | | | | Mice with breast tumours (per cent) |
		1	2	3	4	5	
0–4	50	0	0	0	0	0	0 (0)
5	14	9	1	0	0	0	10 (71)
6	11	4	3	2	0	0	9 (82)
7	14	3	6	0	2	2	13 (93)
8	6	1	1	4	0	0	6 (100)

Over 40 breast tumours were examined histologically   They were adenocarcinomas, often of a papillary type, and frequently with a marked fibroblastic component   A little secretion in the tubules was generally present   Squamous metaplasia was seen in a few tumours, but it was very small in amount   Slight sebaceous metaplasia was sometimes seen

Table IV, V and VI show the relationship between the presence of ovarian secretion (as judged by vaginal smears about the time of death), ovarian tumours (including suspected tumours) and palpable breast tumours χ^2 tests on the data of these tables show a significant correlation between the presence of ovarian tumours and the secretion of ovarian hormones, but not between ovarian tumours and breast tumours, or between breast tumours and ovarian secretion   Only

TABLE IV —*Relative Incidence of Breast and Ovarian Tumours in 45 C57 Bl × IF mice Treated with DMB*

		Breast tumours		
		+	−	Totals
Ovarian tumours	+	27	3	30
	−	11	4	15
Totals		38	7	45

$$\chi^2 = 2.593 \quad P > 0.1$$

TABLE V —*Relation between Presence of Ovarian Tumours and Ovarian Secretion as Judged by Vaginal Smears in 42 C57 Bl × IF Mice Treated with DMB*

		Vaginal smears		
		Oestrus or dioestrus	Anoestrus	Totals
Ovarian tumours	+	21	8	29
	−	3	10	13
Totals		24	18	42

$$\chi^2 = 8.9 \quad P \text{ about } 0.003$$

TABLE VI —*Relation between Presence of Ovarian Secretion and Breast Tumours in 42 C57 Bl × IF Mice Treated with DMB*

		Vaginal smears		
		Oestrus or dioestrus	Anoestrus	Totals
Breast tumours	+	22	13	35
	−	2	5	7
Totals		24	18	42

$$\chi^2 = 2.224 \quad P \text{ about } 0.12$$

mice which survived over 4 months are included, because breast tumours were not apparent before this time

DISCUSSION

Ovarian grafting-experiment

It will be seen from Table I that, in the 10 mice killed 3 months after the first DMB painting, only 1 early tumour was found in serial sections of all 20 ovaries We may be fairly sure, then, that detectable incipient ovarian tumours were present in very few of such ovaries transplanted to the 18 mice in the original experiment (Marchant, 1959a) However, the fact that 14 of them (78 per cent) developed large ovarian tumours indicates that changes leading towards tumour production had already occurred in the majority of ovaries after 3 months treatment The present histological study showed no evidence of any form of hyperplasia, but rather of ovarian atrophy brought about by follicular destruction with accompanying reduction in numbers of corpora lutea In a more recent experiment the mean weight of ovaries from normal young adult F_1 C57Bl × IF mice was

found to be 9 8 mg , while that of ovaries removed 3 months after commencing DMB treatment was only 4 mg

The fact that few really large ovarian tumours were found in the present experiment is undoubtedly due to the time factor involved   Breast tumours developed in rapidly increasing numbers after $4\frac{1}{2}$ months of treatment and this made it necessary to kill all animals by the 8th month   In the original experiment (Marchant, 1959a) the ovaries grafted from DMB treated to normal animals after 3 months of treatment were able to survive a further 14 months in their new hosts, allowing ample time to grow into really large tumours

Histogenesis of ovarian tumours

When we consider the histogenesis of the granulosa-celled tumours, it seems impossible to say what type of tissue the tumours originated from   Fig 13 shows that ovarian tumours steadily continued to appear after 3 months DMB treatment, although by 4 months no follicles remained in any ovaries   It would seem that tumours which arose after 4 months treatment could not have arisen from follicular tissue

There was no evidence of origin of the granulosa-celled tumours from down-growth and invagination of germinal epithelium, such as precedes the appearance of tubular adenomas, described by Russell and Fekete (1958)   The prominence of germinal epithelium and slight invagination of it was only occasionally seen in the late stages of the experiment and seemed to be associated with ovarian atrophy

It is possible that the tumours may have arisen from stem cells in the ovarian parenchyma, a view held by Willis (1953) and others

Luteinisation of granulosa-celled tumours

From a study of the tumours in this and other experiments (Marchant, 1959b) with ovaries from DMB-treated mice, it seems quite clear that luteomas arise from pre-existing granulosa-celled tumours   At the time when granulosa-celled tumours are first detected, the ovaries are usually in a diffusely luteinised condition resulting from fusion of corpora lutea, with fusion and vacuolation of their cells This diffusely luteinised tissue somewhat resembles that of a granulosa-celled tumour that has undergone a marked degree of luteinisation   Thus, on seeing a diffusely luteinised ovary containing an incipient granulosa-celled tumour, one might be tempted to assume that granulosa-celled tumours arise in pre-existing luteomas   That this is not the case can only be determined by study of a series of animals killed at intervals of time throughout the induction period   The series described here has shown that ovaries in a diffusely-luteinised condition are atrophied and do not grow in size, in fact mitosis has never been observed in a luteinised cell

The luteinisation of granulosa-celled tumours was not seen in IF or C57 Bl mice (Marchant, 1957), but it was very marked in the grafting experiment already mentioned (Marchant, 1959a) utilising F_1 C57 Bl × IF mice   It was also seen in the later stages of an experiment in which IF or C57 Bl ovaries were transplanted into C57 Bl × IF hosts prior to DMB-treatment (Marchant, 1959b)   It may be that luteinisation is a matter of maturation of the tumour cells, or it may be brought about by some factor in the internal environment of the hybrid mice differing from that in the parent strains

Hyalinisation

"Hyalinisation" of corpora lutea was a phenomenon not previously encountered in ovaries of C57 Bl mice treated with DMB and only rarely in mice with IF ovaries (Howell, Marchant and Orr, 1954)   It was frequently seen in these C57 Bl × IF hybrid ovaries as in old dba mice (Fekete, 1946) and in dba × CE hybrid mice (Atkinson *et al*, 1954)

Hormone production by ovarian tumours

Secretion of oestrogen, as judged by vaginal smears, was seen in 21/29 (72 per cent) of animals with ovarian tumours (Table V)   This is comparable with the 39/48 (81 per cent) found in the previous report of ovarian tumours in IF or IF hybrid mice (Howell, Marchant and Orr, 1954) and indicates that approximately 1 out of 4 or 5 granulosa-celled tumours in mice does not secrete oestrogen

Breast tumours

The DMB treatment induced breast tumours in the majority of mice surviving more than 4 months   There was no correlation between the presence of breast tumours and oestrogen secretion at the time of death, as shown by Table VI

SUMMARY

Eighty-five virgin female F_1 hybrid mice between C57 Bl and IF strains were used   They were given up to 6 fortnightly skin paintings of 1 mg DMB in olive oil in order to induce ovarian tumours   As far as possible, 10 mice were killed at monthly intervals and their ovaries sectioned and examined histologically

The first change noticed in all ovaries was a gradual diminution in numbers of follicles until all had disappeared by the 4th month   Corpora lutea became fused, until by the 4th month there were rarely any distinct ones to be found   After this, lutein tissue showed considerable variation with frequent vacuolation and pigmentation of cells   Sometimes it became converted to collagenous tissue resembling human corpora albicantia   As a result of these changes the ovaries atrophied in size

Incipient granulosa-celled tumours were found from the 3rd month onwards   They were usually unilateral and were detected in a steadily increasing proportion of animals   About three-quarters of the tumours secreted oestrogen, as judged by vaginal smears   In the later stages the tumour cells showed traces of luteinisation

There was a gradual increase in numbers of palpable breast tumours after the 4th month

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THE INFLUENCE OF PSEUDOPREGNANCY ON THE INDUCTION OF MAMMARY TUMOURS BY METHYLCHOLANTHRENE IN MICE OF THE BALB/c STRAIN

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MAMMARY carcinomas can be induced in virgin IF female mice by means of chemical carcinogens (Bonser, 1958) But it was shown by Bonser (1954) that oestrogen alone was not a sufficient substitute for the ovaries as the hormonal stimulus in the induction of such tumours by methylcholanthrene However, by replacement of the ovarian steroid hormones by oestrogen and progesterone, Jull (1954) was able to obtain 9 tumours in 11 ovariectomised mice He also demonstrated (1953) that virgin females of this strain have a well-developed duct and acinar system, the latter reaching a maximum at about 18 weeks of age but showing much individual variation This extensive acinar development and the demonstration by van der Lee and Boot (1955) that spontaneous pseudopregnancy is frequent in virgins of some mouse strains when the females are caged in groups, suggested that high levels of progesterone might be operating in the IF female and might account for the high incidence of breast tumours induced by chemical carcinogens It was therefore decided to test the effect of repeated induced pseudopregnancies on tumour induction by a chemical carcinogen

The strain chosen was the BALB/c, which does not carry the milk factor (Andervont, 1940) and has a low incidence of spontaneous mammary cancer The only information available regarding the reaction of this strain to chemical carcinogens was that obtained by Squartini (1958) who had failed to induce mammary tumours in 30 virgin mice by means of subcutaneous injection of 1 mg of 20-methylcholanthrene in 0 2 c c of olive oil There was, therefore, no guide in regard to the dose and duration of the chemical treatment to be adopted In order to gain an insight into the mode of action of the chemical, it was decided to test three groups of mice virgins kept five in a cage , virgins deprived of the olfactory lobes and kept singly in order to reduce pseudopregnancy to a minimum (van der Lee and Boot, 1956) , and virgins kept with vasectomised males in order to induce pseudopregnancy

MATERIALS AND METHODS

BALB/c strain

A litter of this strain was given to the Division of Cancer Research, University of Perugia, by the Chester Beatty Research Institute, London, in November, 1953 It was then in the 79th generation of inbreeding The Chester Beatty

Institute had previously obtained the strain from L Dmochowski, Department of Cancer Research, Leeds, in December, 1952, when it had reached 76 inbred generations   The donor to Leeds was H B Andervont

During the period in Perugia, this strain has reached the 99th generation of inbreeding and has shown a low mammary cancer incidence   During 1958, in 10 virgins there were no tumours and in approximately 40 breeders there were 7 5 per cent the latent period being 76 weeks or more   During 1959, no tumours occurred in 44 virgins, but in 44 breeders the incidence was 7 0 per cent at the same late date   In the hands of Andervont (1941) this strain had an incidence of mammary cancer in breeding females of less than 2 per cent

Group I (36 mice)—At 4–5 weeks of age, virgin mice were placed five in a cage and were so kept throughout the experiment

Group II (41 mice)—At approximately 6 weeks of age, under ether anaesthesia, the olfactory lobes were removed surgically from virgins by means of suction through small trephine holes in the anterior part of the cranium immediately on either side of the mid-line   The mortality was low (10 per cent) After recovery the mice were kept five in a cage for approximately one week, but thereafter they were placed singly in cages

Group III (32 mice)—At 6 weeks of age, groups of three virgins were mated with one vasectomised male, which was allowed to remain in the cage throughout the experiment

All groups received similar chemical treatment, namely six applications to the skin at fortnightly intervals of 16 drops of 0 5 per cent 20-methylcholanthrene (supplied by Messrs Light & Co Ltd, Colnbrook, Bucks) in almond oil (8 drops on the dorsal and 8 on the ventral surface) commencing at 12 weeks of age   It was computed that 1 ml of oil, containing 5 mg of carcinogen, was used for each application and that the mice were thus exposed to the carcinogen for a period of 12 weeks   The animals stood the treatment well

A diet of cubes (supplied by Messrs Pilsbury, Birmingham) and water *ad lib* was given

At post mortem a whole mount was prepared of the third left (thoracic) breast of each treated mouse   The ovaries with capsule were weighed wet and the uterine horns were assessed by naked eye examination as normal, increased or decreased in size

RESULTS

Mammary tumours

(a) *Incidence*—The date of appearance of the mammary tumours and the survival of the non-tumourous mice are shown in Fig 1   All the tumours were single, except in two mice of Group III   In one of these, three tumours developed at 25 weeks and in the other two tumours at 37 weeks   In Table I the percentage of mice bearing mammary tumours in relation to survival after the initiation of treatment is given   No tumours occurred in virgins (Group I), 2 4 per cent in lobectomised virgins (Group II) and 43 8 per cent in females mated with vasectomised males (Group III)   The survival rate was considerably shorter in the last group, 4 of 32 mice surviving for more than 32 weeks, whereas the survival was 35 out of 36 and 33 out of 41 in the other two groups respectively (Fig 1)
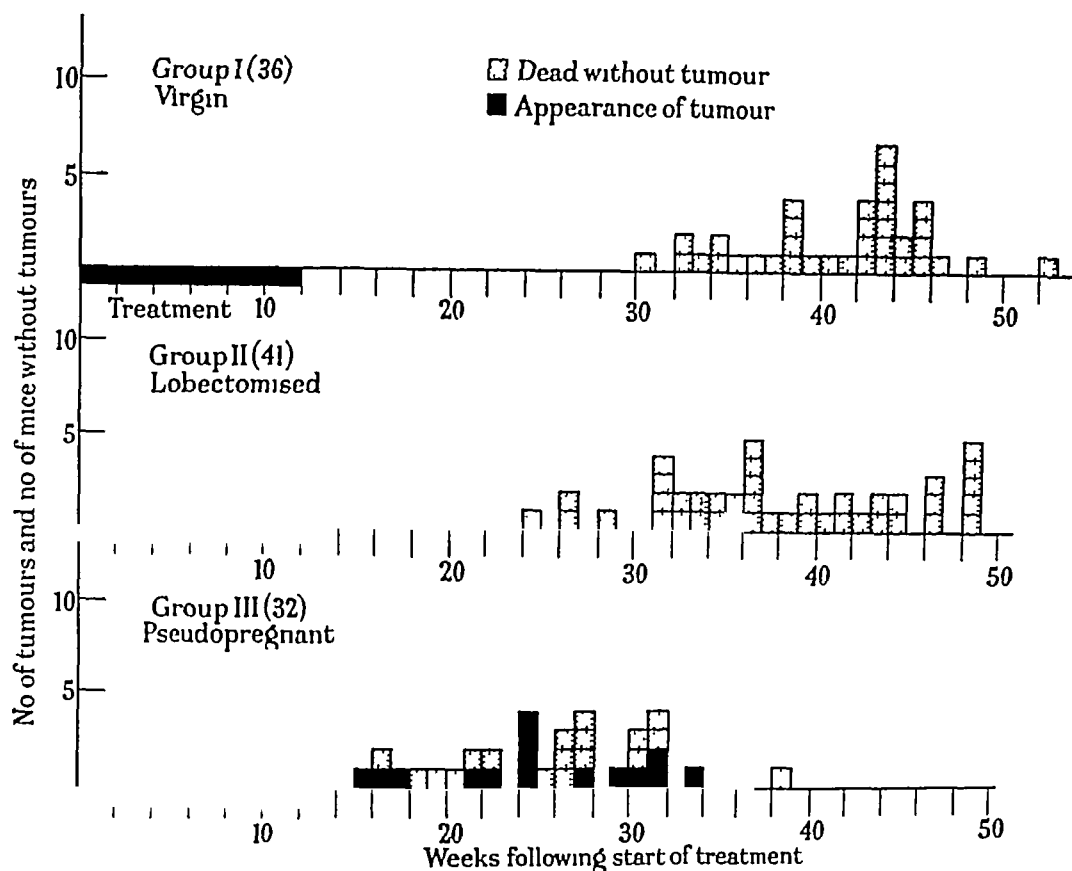
FIG 1 —Incidence of mammary tumours

EXPLANATION OF PLATES

FIG 2 —Irregular tubular mammary carcinoma (Group III) appearing 25 weeks after the beginning of treatment The tubules are irregular in size and shape and there is little stroma except at bottom right   × 60

FIG 3 —Solid polygonal cell mammary carcinoma (Group III) appearing 22 weeks after the beginning of treatment, with advanced squamous metaplasia   × 60

FIG 4 —Papillary cystic mammary carcinoma (Group III) appearing 32 weeks after the beginning of treatment   × 60

FIG 5 —Whole mount of third left breast (Group I) 31 weeks after the beginning of treatment A well developed pattern of dilated ducts is seen, with no lobules but numerous end buds   × 60

FIG 6 —Whole mount of third left breast (Group III) 24 weeks after the beginning of treat ment Florid acinar development This mouse had a mammary tumour in another breast   × 60

FIG 7 —Whole mount of third left breast (Group III) 21 weeks after the beginning of treat ment Nodule at top   × 60

FIG 8 —Ovary (Group I) 41 weeks after the beginning of treatment Atretic follicles round the edge Remains of corpora lutea in the interstices with a group of dark staining cells in the centre Pigment containing phagocytes below centre and top right   × 60

FIG 9 —Ovary (Group III) 27 weeks after the beginning of treatment Atretic follicles top right and bottom left Numerous large, old corpora lutea   × 60

FIG 10 —Longitudinal section of uterine horn (Group I) 45 weeks after the beginning of treatment Lumen narrow with simple crypts dipping into stroma and simple tubular glands   × 60

FIG 11 —Longitudinal section of uterine horn (Group III) 27 weeks after the beginning of treat ment Lumen irregular with irregular crypts dipping into stroma Large number of glandular cross sections   × 60
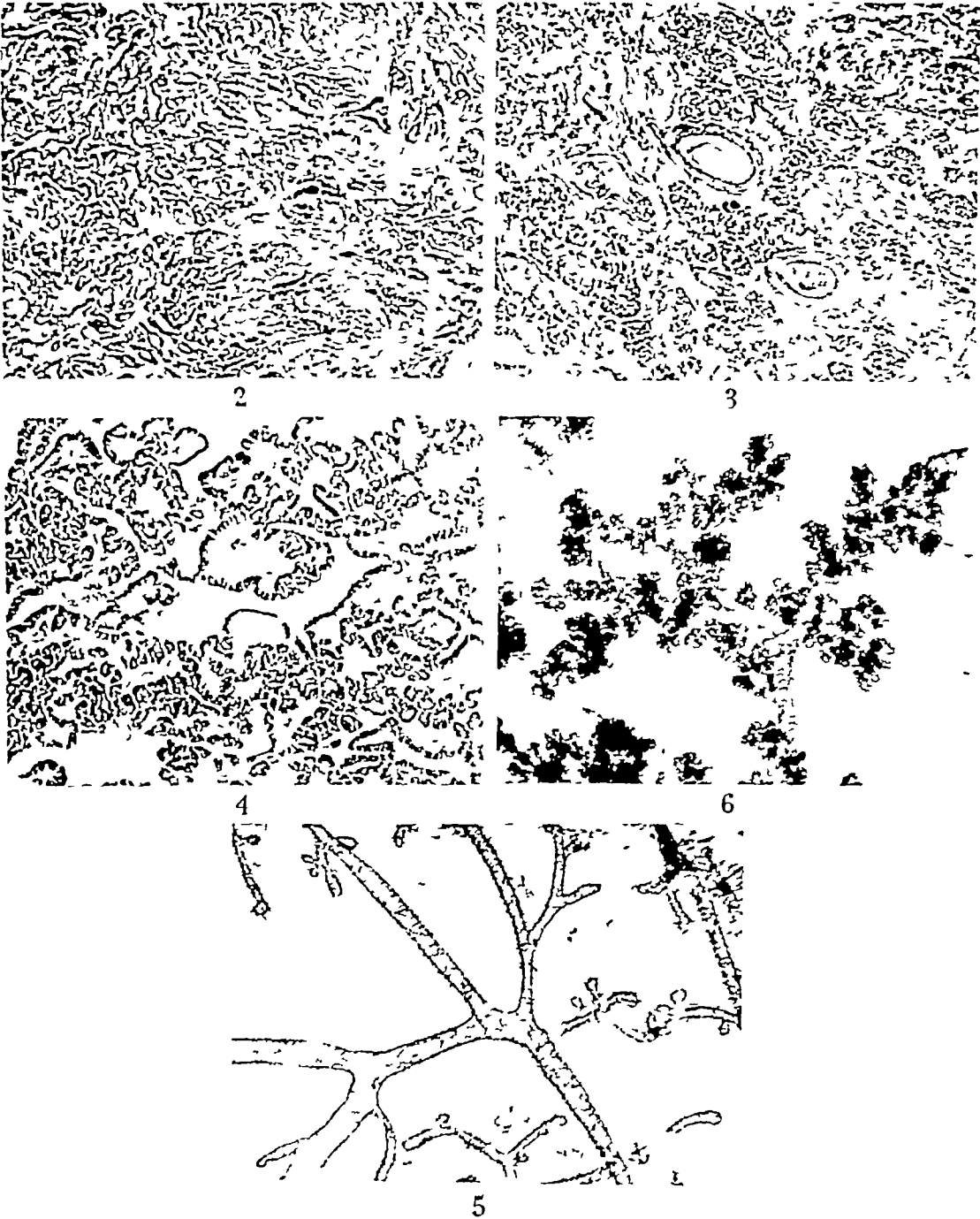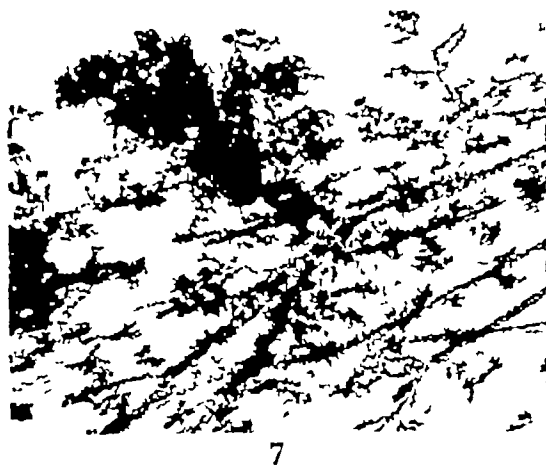
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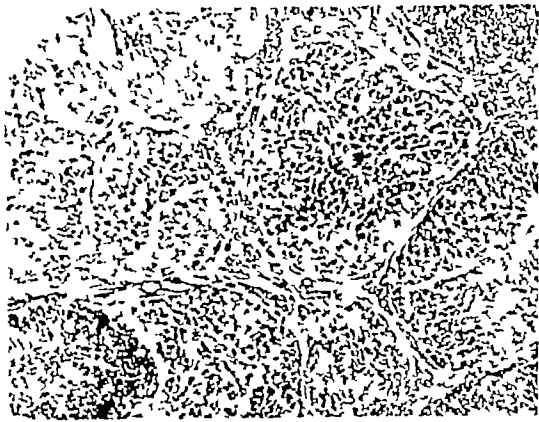
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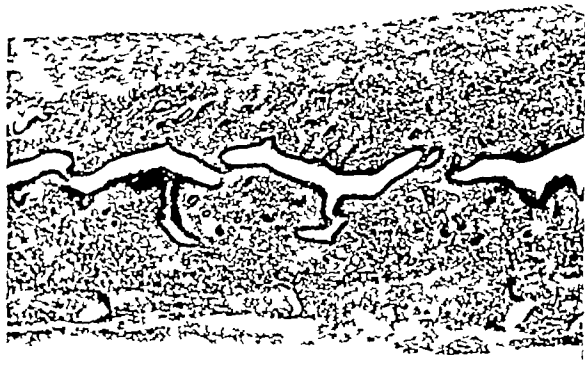
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TABLE I —*Incidence of Mammary Tumours in Mice Surviving to the Beginning of Each Ten-week Period*

	Weeks of survival following initiation of treatment						Tumours
Group	10–19	20–29	30–39	40–49	50–59	Total	(per cent)
I	36	36	36	22	1	0/36	0
II	41	41	1/37	18	0	1/41	2 4
III	3/32	5/29	6/11	0	0	14/34	43 8

Numerator = number of mice bearing mammary tumours
Denominator = number of mice surviving to the beginning of the stated period

(b) *Histological structure* —Using the classification adopted by Bonser (1954), the mammary tumours in Group III were classified as follows (Fig 2–4) irregular tubular 11 (6 with squamous metaplasia and five without), solid polygonal cell four (all with squamous metaplasia), and two papillary cystic tumours (one with early squamous metaplasia) No carcinosarcomas occurred The one tumour which appeared in Group II was of solid polygonal-cell type, with squamous metaplasia Emboli of tumour cells were present in some of the perivascular lymphatics of one lung

Structure of the breast

This was assessed by the examination of the whole mounts No differences could be detected in the breasts of Groups I and II The duct system was well developed, individual ducts were generally slightly dilated, subsidiary ducts were small in number, end buds were prominent but acini were few (Fig 5) and only in two mice were small lobules seen In two other mice single " nodules " were seen By contrast, the breasts of Group III showed florid acinar development, the main ducts were often greatly dilated (especially towards the nipple) and nodules were present in all but one of the mice (Fig 6 and 7) The latter were numerous, except in 4 mice in which one or two nodules only occurred

Skin Tumours

Squamous papillomas and carcinomas of the skin occurred very frequently in any site in all groups The larger carcinomas usually ulcerated and thus reduced the life span of the animal

Lung tumours

Pulmonary adenomas, usually multiple, occurred in all groups usually remaining small in size

Ovaries

(a) *Weight* —Those of Group III were significantly heavier than those of the other two groups (Table II) The range in all groups was considerable

(b) *Histological structure* —There was a marked difference in the appearance of the ovaries in Groups I and II compared with Group III In the former, atretic follicles in various phases and broken-up remnants of corpora lutea were seen (Fig 8) Entirely normal follicles were absent, though some young follicles

TABLE II —*Ovarian Weights and Uterine Volume*

Group	Ovary			Uterus			
	Number of mice	Average (mg)	Range (mg)	Number of mice	Increased volume	Normal volume	Decreased volume
I	36	0 011	0 003–0 027	36	10	10	16
II	41	0 010	0 003–0 020	41	2	8	31
III	32	0 018	0 009–0 030	32	26	5	1

showed only a damaged ovum   Corpora lutea were present only in occasional mice of Group I   In the ovaries of Group III, atretic follicles were present in numbers approximating to those in the other two groups, as well as large numbers of large corpora lutea (Fig 9)   The lutein cells stained dark pink with eosin and were judged to be old structures   Although occasional luteomatous proliferations were seen in the ovaries of all groups, no ovarian tumours were detected

Uterus

(a) *Volume* —The uterus was inspected at post mortem and an assessment was made of the size of the horns (Table II)   In Group I the horns were either thread-like, normal or slightly increased in volume , in Group II they were usually thread-like, whereas in Group III they were often thick and dilated

(b) *Histological structure* —The uteri of Groups I and II had a structure characteristic of the virgin mouse, the endometrium being composed of a single lining layer of columnar cells placed on dense stroma, into which dipped small numbers of simple tubular glands   The lumen was narrow, with occasional crypt-like depressions into the stroma (Fig 10)   The uteri of Group III showed a very different picture   There was no excess of stroma and the component cells were not swollen, but the amount of surface epithelium was greatly increased by numerous irregular clefts which dipped into the stroma and gave the lumen a papillary aspect (Fig 11)   The number of cross-sections of tubular glands was increased, due either to an increase in the actual number of glands or to a more complicated structure of those present   Penetration of the endometrial glands into the muscular coat did not occur

DISCUSSION

The present experiments have demonstrated that intact or lobectomised virgins of the BALB/c strain, which is free of the milk factor and has a low spontaneous incidence of mammary cancer, do not develop mammary tumours when treated with a dose of methylcholanthrene which is known to be in excess of that required to induce tumours in virgins of the IF strain   Jull (1956), using a standard limited dose of 4 applications to the skin of an oily solution of the compound, obtained 6 mammary tumours in 16 IF mice (38 per cent)

When pseudopregnancy was induced by mating females with vasectomised males (Group III) 44 per cent developed mammary tumours, the induction period ranging from 16–37 weeks following the initiation of treatment, a result closely comparable to that obtained by Jull in IF virgins   Thus the hormonal conditions of pseudopregnancy act as a promoting agent for the induction of mammary tumours in BALB/c breasts already submitted to the initiating action of a carcin-

ogen This type of promoting stimulus is not essential for the induction of mammary tumours in this strain by the milk factor for Severi, Olivi and Bianci- fiori (1958) found an incidence of 54 per cent of mammary tumours in *virgins* at an average age of 50 weeks in BALB/c mice which had been given milk factor by Andervont in 1940 (called BALB/c+ in Perugia)

The florid structure of the breasts, the large number of corpora lutea in the ovaries and the hyperplastic state of the endometrium constitute evidence that the mice placed with vasectomised males were under the influence of oestrogen- progesterone secretion Thus these experiments provide further evidence that the carcinogenic action of methylcholanthrene on the mouse breast is augmented by oestrogen combined with progesterone

The absence of tumours in intact and lobectomised virgins was not due to short survival in these groups, for they lived a good deal longer than the pseudo- pregnant mice (Fig 1) It might be suggested that pseudopregnancy alone was the cause of the mammary tumours and that initiation by the chemical was an unnecessary factor This seems unlikely, as mammary tumours occur spontane- ously in low yield only, in old breeders of this strain It is a point which is, however, under investigation

No attempt was made to study the histogenesis of the mammary tumours in Group III but the presence of large numbers of typical hyperplastic nodules in the breasts is in keeping with van Rijssel's (1956) demonstration that in mice with the milk agent there is a relation between the number of these structures and the subsequent appearance of the fully formed tumours He calculated that about 60 nodules were necessary for every palpable tumour that presented

The morphology of the induced tumours was similar to that of chemically- induced tumours of the IF strain The predominant tumour was the irregular tubular adenocarcinoma, which may be regarded as the characteristic tumour of chemical induction, but solid polygonal and papillary cystic tumours also occurred In two thirds there was squamous metaplasia, thought by Bonser (1958) to be associated with excessive dosage of the chemical Although carcinosarcomas were not found, this type of tumour is not uncommon in IF mice treated with methylcholanthrene or 1 2 5 6-dibenzanthracene

SUMMARY

Three groups of females of the BALB/c strain (with low breast cancer inci- dence and no milk factor) were treated with 20-methylcholanthrene applied to the skin in oily solution

In Group I (36 virgins kept 5 in a cage) no mammary tumours occurred, although the mice survived for a period greater than 30 weeks after the beginning of treatment In Group II (41 virgins with olfactory lobes removed) there was one mammary tumour 36 weeks following the initiation of treatment, 37 mice having survived for 30 weeks or more In Group III (32 females kept 3 in a cage with a vasectomised male) the incidence of mammary tumours was 43 8 per cent, the latent period being 16–37 weeks Benign and malignant tumours of the skin and lung adenomas occurred in all groups

In the mice of Group III there was evidence of the excessive hormonal stimu- lation of pseudopregnancy in the florid structure of the breasts, the large number of corpora lutea in the ovaries and the hyperplasia of the uterine endometrium

It is postulated that the hormonal stimulation of pseudopregnancy, through oestrogen and progesterone, acted as the promoting agent in the causation of mammary tumours in breasts which had been subjected to the initiating action of methylcholanthrene

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TISSUE REGENERATION AND TUMOUR DEVELOPMENT

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THE early changes, including hyperplasia, induced in the liver of the rat by the oral administration of 2-acetylaminofluorene were described by the author and his French colleagues (Laws, Mabille, Royer and Rudali, 1952) and in less detail by Skoryna and Webster (1951) The studies have been continued for some years in this department Recently Laird and Barton (1959) have investigated the quantitative aspect of this effect and have pointed out that the period up to the onset of hyperplastic change in the liver of animals treated with this carcinogen appears to coincide with the minimum period of administration of the chemical which is necessary to provoke the ultimate appearance of tumours in this organ The work described here has made use of the possibility of inducing regenerative hyperplasia in the liver by means of partial hepatectomy to study in more detail the influence of such a reaction on the effect of 2-acetylaminofluorene Histological examination of the liver during the process has helped to make clear some of the mechanisms involved

MATERIALS AND METHODS

Animals —The rats used in most of this work were bred in this laboratory and were of Wistar stock In the experiment contrasting the behaviour of young and old rats, the animals were of Birmingham stock, obtained from the Department of Anatomy, University of Birmingham No difference has been noticed in the response of these two stocks of rats to treatment with 2-acetylaminofluorene Only male rats were used throughout, since liver changes are rarely found in females Unless noted all rats were adult (weight at least 200 g) when the experiments began

Carcinogen treatment —The carcinogen, 2-acetylaminofluorene was obtained in pure form from L Light and Sons It was used, at a strength of 0 1 per cent incorporated in a meal diet supplied by the North Eastern Agricultural Co-operative Society, Aberdeen The carcinogen was dissolved in 500 ml of acetone and mixed with 10 kg of the meal which became lightly dampened throughout. Water was added to form a paste which was formed into cakes and dried These were fed *ad libitum* to the rats together with water The meal used was of the same composition as the rat cakes from the same manufacturers used to feed the general stock of rats and mice, and provided a full balanced diet

Partial hepatectomy —The operation, by the method of Higgins and Anderson (1931), consisted of the removal of the anterior and left anterior lobes of the liver after ligature Approximately two-thirds of the liver substance was effectively removed, on the average

General —All animals included in the experimental figures were subjected to a full post-mortem examination and histological sections of all relevant lesions were examined after staining with haematoxylin and eosin

RESULTS

The earliest changes provoked by 2-acetylaminofluorene are of a toxic nature, reducing the efficiency of the liver cells and leading to the death of many cells In earlier experiments of the author (Laws *et al*, 1952) and in those of Laird and Barton (1959) this was in some cases sufficient to lead to the death of the animals Even in those rats less severely affected, histological examination of the liver showed a loss of parenchymatous cells around the portal tracts, with hyperplasia of small non-parenchymatous cells of uncertain origin This stage is followed by one in which cell division among the parenchyma cells rapidly produces a replacement of the lost tissue, and goes on steadily to genuine hyperplasia In the animals used in the present experiments (albino rats of so-called Wistar strain bred in the laboratory, but of uncertain ancestry), the changes are less dramatic, death seldom occurring during normal 2-acetylaminofluorene feeding Histologically also the changes are more gradual but the outcome is the same, a nodular, hyperplastic liver In spite of the little apparent change in the livers of these animals, partial hepatectomy performed after three weeks feeding unmasks a profound diminution in liver efficiency If such animals are treated with the carcinogen after the operation also, most of them are dead within two weeks (26 out of 40 animals up to date) Such animals which die or are killed at this time are found to have liver weights only about one-third of the normal, i e only about the amount of liver left by the hepatectomy, in contrast with the rapid replacement of liver tissue in normal animals after such an operation Ascites is invariably present and usually other effusions and a general waterlogging of the tissues This probably results from hypoalbuminaemia due to poor liver function, although this has not so far been proved by direct estimation

Histologically such livers present the series of changes noted above in a very severe form Within a few days of the operation much of the parenchyma of the remaining lobes has disappeared, principally around the portal tracts, and there is marked proliferation of small cells in this region, stretching out into the parenchyma which is still present (Fig 3) If the animal survives as long as a week, regeneration begins and can be seen by the eighth day by the naked eye in the form of small translucent nodules on the surface of the organ, and on the cut surfaces Such nodules can be shown by a series of preparations examined histologically to originate as small groups of cells (presumably formed in the first place by division of one cell) staining basophilically and containing many mitotic figures (Fig 1 and 2) They are always situated in the region of the radicles of the hepatic vein, the part least damaged, although they enlarge rapidly and by the fourteenth day may largely have replaced the remaining old parenchymatous cells, and the small cells which had infiltrated (Fig 4) The animal may die even at this stage but if it survives, the enlargement continues to reproduce the original bulk of the liver and beyond this to genuine hyperplasia

Hepatectomy performed five weeks after the commencement of carcinogen feeding leads to similar results if the administration is continued after operation After seven weeks of feeding however the mortality is much less, and the animals

remain healthier   Liver regeneration is still slow in such animals, but the organ weight reaches the lower limits of normal in some by the fourteenth day   Microscopically such animals show less destruction after operation and a more rapid onset of the nodular regeneration, otherwise similar in type   At the other extreme, if hepatectomy is performed on the day on which feeding with 2-acetylaminofluorene is started there is little delay in the onset of a normal general regenerative reaction, and the liver weight returns to near the normal limits in seven days   There is however the tendency for the weight to remain a little below normal, and nodules start to form as in the other cases   By the fourteenth day such nodules containing many mitotic figures are prominent   Regeneration in animals given three weeks treatment with carcinogen and then two weeks on a normal diet before hepatectomy behave much like those given no treatment before operation   This suggests that an extreme degree of liver damage, likely to cause death after hepatectomy, is due to cumulative toxic action on the liver cells by the 2-acetylaminofluorene   Such an extreme degree of damage is not a prerequisite for the onset of nodular proliferation, although this process is undoubtedly more vigorous in animals threatened with imminent decease from liver failure   It does appear that some degree of " strain " precipitated by functional incapacity, is needed to start this process going   How such a " strain " can be translated into a stimulus which results in the production of a new race of liver cells capable of resisting the toxic effects of 2-acetylaminofluorene, and of still responding to the physiological stimulus for regeneration, remains to be investigated

So many of the changes which accompany carcinogenesis in any organ must be incidental that to establish a connection between such events and the process of carcinogenesis itself it is essential to show that they do affect the actual production of tumours   In the case under consideration the performance of a hepatectomy at the appropriate period might be expected to alter the latent period or the incidence of liver tumours   It might also shorten the period of administration of carcinogen needed to produce tumours, if the precocious appearance of the changes described, means that the " initial, essential step " (Laird and Barton, 1959), has already been accomplished at the end of three or four weeks   Experiments already carried out by the author (Laws, 1956) have established an effect of hepatectomy, carried out at the beginning of the carcinogen feeding period, on the latent period of liver tumour production, although the incidence of tumours was unaltered   In the first experiment (Table I), animals treated by this pro-

TABLE I

		Hepatoma incidence in periods			
		(a) 12–14 months	(b) 15–17 months	(c) 18–24 months	
Group	Hepatectomy performed	(after start of AAF feeding)			Total
A	None (normal controls)	1	6	1	8/11
B	3 weeks before AAF feeding started (Hepatect controls)	0	5	3	8/10
C	At start of AAF feeding	5	4	1	10/10
D	After 3 months of AAF feeding	0	1	8	9/11
E	At end of AAF feeding (4 months)	1	3	4	8/10

(Total out of number alive at twelve months)

cedure showed five deaths with gross liver tumours out of ten animals at a time when only two out of forty-two other rats not hepatectomised early in the feeding period had developed tumours   These other animals included, as well as normal controls, animals hepatectomised after three and four months of carcinogen feeding, that is to say after the onset of hyperplasia   In these latter animals the onset of tumours was, if anything, delayed beyond the period seen in the normals   Skoryna and Webster (1951) also concluded that partial hepatectomies started in the third month of 2-acetylaminofluorene feeding, even if repeated, did not accelerate the appearance of tumours   A further experiment on these lines is still in progress , the first results are similar to those of the earlier one

The period by which the appearance of tumours was accelerated was about three months, which coincides roughly with the period taken in our animals for the appearance of hyperplasia on plain 2-acetylaminofluorene feeding   This would fit in with the suggestion of Laird and Barton (1959) that it is from the onset of hyperplasia that the irreversible process of tumour formation should be dated   It would seem that the latent period before the onset of rapid tumour growth is unaffected by hepatectomy outside the " critical " period, which lasts up to the time of onset of hyperplasia during 2-acetylaminofluorene feeding   The finding of Glinos, Bucher and Aub (1951) that in rats treated with dimethyl-aminobenzene, hepatectomy at the time of withdrawal of the carcinogen appeared slightly to hasten the appearance of tumours, suggests that the mechanism of action of the azo-dye carcinogens may differ from that of 2-acetylaminofluorene   This is made more probable by the difference in the time-course of liver hyper-plasia noted by Laird and Barton when using a carcinogen of the azo-dye group (3'-methyl-dimethylaminoazobenzene)

These authors also note that the onset of hyperplasia occurred later in rats which were of greater weight, and incidentally older, at the time carcinogen feeding was started   In another experiment carried out by the author in this laboratory it has been shown that the latent period before the onset of active tumour growth is also affected by the age of the rats at the time at which feeding with carcinogen is started   Two groups of rats were given the same course of four months feeding with 2-acetylaminofluorene   The first group had an average body weight of 100 g and the second an average of 380 g at the start   The small rats gained weight during the four months in spite of treatment, the larger animals lost weight   The results, not previously reported, show a significantly earlier incidence of tumours in the young group (Table II)   By fourteen months there had been six

EXPLANATION OF PLATE

FIG 1 —Rat 1   Wt 260 g   Wt of liver at death 4 5 g (normal 10 g)   Killed 8 days after partial hepatectomy   Infiltration of cells round portal tracts and basophilic nodules near central veins   × 110
FIG 2 —Rat 1   High power view of basophilic nodule with mitosis   × 550
FIG 3 —Rat 2   Wt 260 g   Wt of liver at death 2 75 g (normal 10 g)   Found dying nine days after partial hepatectomy   Severe infiltration round portal areas with diminution of parenchyma   Basophilic nodules only beginning to form   × 65
FIG 4 —Rat 3   Wt 360 g   Wt of liver at death 3 5 g (normal 13 g)   Found dying 14 days after partial hepatectomy   Liver contained many pale or translucent areas on naked-eye examination   Nodules have enlarged and are compressing the surrounding original tissue and the infiltrating cells   × 65
(All animals received carcinogen treatment for three weeks before hepatectomy, and subse quently until death)
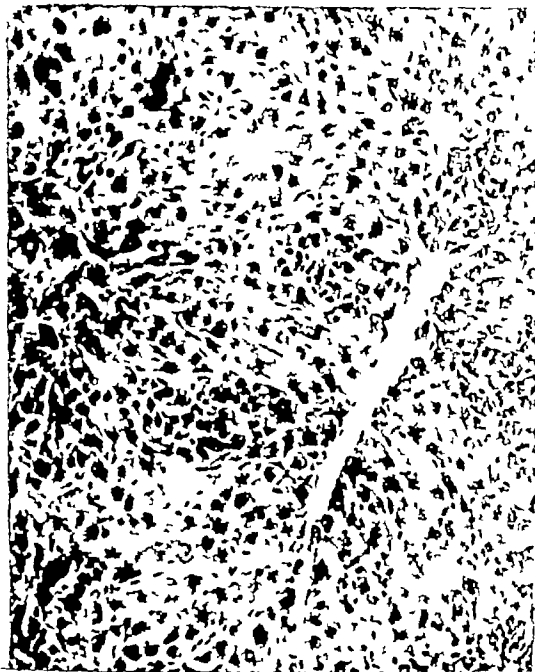
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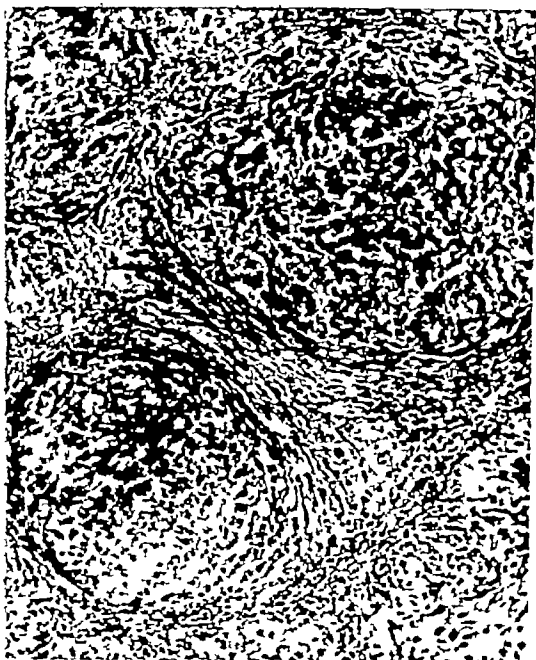
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TABLE II

Hepatoma incidence in periods

	(a) 12–14 months	(b) 15–16 months	(c) 17 months and over	
Group	(after start of AAF feeding)			Total
Young (100 g body wt)	6/7 (i e 1 death without hepatoma)	0/1	2/2	8/10
Old (380 g body wt)	1/4 (i e 3 deaths without hepatoma)	0/3	3/4	4/11

(Hepatoma deaths given as fraction of total deaths)

deaths with large liver tumours in this group, but only one in the older animals Later tumours appeared more frequently in the older group Comparison of the total tumour incidence in the two groups is unsatisfactory since many of the older animals died before reaching the period of highest tumour incidence in this group There was a difference of about ten weeks in the median tumour death ages for the two groups, which is similar to the difference in the time of onset of hyperplasia noted by Laird and Barton in their experiments with groups of rats of different initial weights, using this carcinogen

DISCUSSION

Following partial hepatectomy in rats which have received 2-acetyl-aminofluorene, mitotic activity occurs only in the hyperplastic nodules which then appear Even when the nodules are still small, mitoses have not been seen in the sickly parenchyma around them Once a nodule has formed mitosis occurs rapidly, and the eventual hyperplastic liver has apparently been formed from the descendants of the comparatively small number of cells which have originated the nodules This process contrasts with the diffuse mitotic activity seen in normal regeneration, and which is reported by Laird and Barton (1959) as occurring in rats treated with 3-methyl-dimethylaminoazobenzene For this reason it would seem unwise to compare directly results obtained with the two carcinogens, or to assume that quantitative results obtained with one can be applied to a consideration of the mode of action of the other Under the conditions of nodular replacement obtaining in the 2-acetylaminofluorene treated animals, regenerative activity is of course slower The time taken in the experiments for the return of the liver weight to normal levels is sufficiently slow to allow for the extra generations of cells which must be produced Observations on the similar, though more gradual formation which occurs in uncomplicated carcinogen feeding suggests that here too there is a gradual replacement of " normal " liver cells by the descendants of a small number of cells which have been changed in some way by exposure to the carcinogen In particular, as suggested by Skoryna and Webster (1951), they appear to have become resistant to the toxic action In spite of this suggestion that all the cells in a hyperplastic liver are " changed ", tumours arise from only a minority of the cells by a further, later burst of mitotic activity accompanied by other changes in behaviour It seems that as Laird

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and Barton (1959) suggest, an essential step for carcinogenesis occurs when hyperplasia supervenes   With 2-acetylaminofluorene further alterations, perhaps not dependent on the direct action of the carcinogen, are necessary before true tumour formation can occur, as well as subsequently   Further the possibility must be considered that the change in certain cells which results in a resistance to the toxic action of the carcinogen and which enables hyperplasia to occur, may not necessarily be the essential carcinogenic change   It may accompany this, or set the stage for it   Experiments at present in progress on the behaviour of cells isolated from hyperplastic livers into tissue culture may help to throw some further light on the changes which occur at this stage, as may others in which a short period of carcinogen feeding, too short to give rise to tumours normally, is being combined with hepatectomy at the second or third week of feeding

SUMMARY

(1) Liver regeneration in rats following hepatectomy is severely delayed and disorganised when the carcinogen 2-acetylaminofluorene is administered for three weeks before the operation and subsequently   Regeneration under these circumstances occurs by nodular proliferation from a relatively small number of centres

(2) When hepatectomy is performed at the beginning of the carcinogen feeding period, the initial phase of regeneration proceeds normally but is quickly superseded by nodular replacement   Such animals show a reduction in the latent period for liver carcinogenesis

(3) Rats started on a course of 2-acetylaminofluorene treatment at the age of eight weeks also show a reduced latent period for liver carcinogenesis as compared with rats which are adult at the start of the treatment

(4) It is suggested that during such treatment with 2-acetylaminofluorene an irreversible step in the process of carcinogenesis occurs at the onset of nodular hyperplasia   Hepatectomy early in the feeding period probably brings this step about precociously

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THE EFFECT OF TISSUE OXYGEN TENSION ON THE RADIO-SENSITIVITY OF LEUKAEMIA CELLS IRRADIATED *IN SITU* IN THE LIVERS OF LEUKAEMIC MICE

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A DETAILED description of the lymphocytic type of leukaemia of CBA mice used in the present study, including an assay method for determining the density of viable leukaemia cells in single-cell suspensions prepared from the infiltrated livers of fully leukaemic mice, was given in a previous paper (Hewitt, 1958) The assay method was later used to determine a survival curve for the liver leukaemia cells irradiated *in vivo* in leukaemic mice breathing air during total-body irradiation (Hewitt and Wilson, 1959) Under these conditions a linear relationship was demonstrated between whole-body radiation dose and log survival ratio among the viable leukaemia cells The D_0 value given by the linear part of the curve (the increment in dose of radiation required to reduce the number of viable leukaemia cells to 37 per cent) was 165 r ^{60}Co gamma radiation The disposition of the points suggested a 2-hit curve but this feature was not determined with certainty Comparison of the D_0 value for mouse leukaemia cells with the D_0 value obtained for human HeLa cells irradiated *in vitro* under well oxygenated conditions (Puck and Marcus, 1956) could not be usefully made without information concerning the oxygen tension in the environment of the mouse leukaemia cells at the time of their irradiation *in vivo* in mice breathing air Comparison of the radiosensitivities of the HeLa cells and mouse leukaemia cells, as described by the established D_0 values, would only be valid if it could be shown that the leukaemia cells were, like the HeLa cells, in a well-oxygenated environment at the time of their irradiation The importance of this comparison relates to the appropriateness of extrapolation to human tumours of radiobiological data obtained for mouse tumours

The macroscopic and histological appearances of the livers of the fully leukaemic mice used for determination of the *in vivo* survival curve already referred to suggested that the vascularity of the liver is impaired at this stage of the disease , and it was considered that an unknown proportion of the masses of vigorously metabolising leukaemia cells infiltrating the liver might have been under severely hypoxic, if not anoxic, conditions at the time of irradiation If this proportion were large, a rise in the radiosensitivity of the cells might be expected if the mice were exposed to radiation while breathing oxygen instead of air , on the other hand, rendering the cells anoxic during irradiation, by killing the mouse before exposure, would not be expected to reduce the radiosensitivity significantly Investigations into these questions form the subject of the present paper

MATERIALS AND METHODS

Mice —CBA male mice bred in this laboratory by brother-to-sister mating were used in all the experiments , the mice were 2–4 months old at the time of experiment   The leukaemic mice which were irradiated had been injected intraperitoneally with several million leukaemia cells 9–11 days previously   At the time of irradiation the mice were moderately sick, almost all organs being heavily infiltrated with leukaemia cells

Irradiation of mice —The leukaemic mouse was placed in a " Perspex " cylinder of such dimensions as permitted the mouse to assume a normal uncramped resting posture but prevented it from turning round   The cylinder was closed at each end with a rubber bung perforated by a short length of glass tubing, one end of which served as an exit for the gas mixture to be circulated through the cylinder The gas mixture was allowed to flow in at the opposite end via a flow meter from a cylinder containing the desired gas mixture (British Oxygen Company, Ltd) Two gas mixtures were used   air containing 5 per cent carbon dioxide, and oxygen containing 5 per cent carbon dioxide   The carbon dioxide was included to ensure an adequate respiratory stimulus   Each gas mixture was allowed to flow through the cylinder at a rate of 1 8–2 2 litres/min for 10 minutes before, and throughout, irradiation   The " Perspex " cylinder containing the mouse to be irradiated was positioned in a beam of ^{60}Co gamma radiation from a Kilocurie beam unit (a " Theratron ")   The whole-body dose was delivered at a mean dose rate of 70–74 r/min and was given as equal exposures to both sides of the cylinder   The distance from the source to the centre of the cylinder was 62 cm and the field size used was such as to cover the mouse very generously   Under these conditions, the whole-body dose was uniform throughout the mouse to about ± 3 per cent

At each radiation dose level used, an air-breathing and an oxygen-breathing mouse, both at the same advanced stage of the disease, were exposed separately but on the same day, the two mice being treated under identical conditions except for the different gas mixtures respired   Experiments at different dose levels were done on different days, but mice at a similar stage of the disease were used on all occasions

For irradiation of the leukaemia cells under what are assumed to be anoxic conditions leukaemic mice were killed by fracture of the neck 1 minute before the start of their exposure to radiation under the same conditions as the living mice The series of dead mouse experiments was undertaken at a slightly later stage of the leukaemia's history than the living mouse experiments   However, the radiosensitivity of the cells in living mice was determined again after completion of the dead mouse experiments, and was found to be unchanged

Measurement of the survival ratios in irradiated leukaemia cell populations — Details of the method of preparing single-cell suspensions of leukaemia cells from the livers of leukaemic mice have been described previously (Hewitt, 1958) In the present experiments, such suspensions were prepared from the livers of the irradiated mice within 20 minutes of the end of their exposure   The density of morphologically intact, and *apparently* viable, leukaemia cells was determined by counting in a haemocytometer by phase-contrast microscopy   0 2 ml volumes of serial tenfold dilutions of the counted suspension in 5 per cent CBA mouse serum in Tyrode solution were injected intraperitoneally into groups of

6 CBA male mice   The range of mean cell doses injected was preselected to cover the expected end-point of an assay   The injected mice were observed for a period of 90 days (a period twice as long as the longest latent period ever observed in a mouse injected with a small inoculum of cells of this strain of leukaemia), and the incidence of leukaemic deaths was recorded for each group   From the results, the number of morphologically intact leukaemia cells required to transfer leukaemia to half a group of injected mice was calculated by the method of Reed and Muench (1938)   It was found that the yield of morphologically intact leukaemia cells obtained from the livers of irradiated mice within one hour of irradiation was not reduced below that expected from untreated leukaemic mice   The TD50 values obtained for morphologically intact cells from irradiated mice, however were significantly higher than the average value given by cells from untreated mice and were a function of the dose of radiation   Thus, irradiation abolished the reproductive integrity of a proportion of the leukaemia cells without producing immediate morphological changes appreciable by phase-contrast microscopy   It was found previously (Hewitt, 1958) that in 6 assays of leukaemia cells from unirradiated leukaemic mice, the TD50 values varied from 0 7 to 3 0 cells, averaging 2 0 cells   The log survival ratio in an irradiated leukaemia cell population was calculated simply by subtracting the log TD50 given by the irradiated cell population from the log of the average TD50 given by unirradiated populations

RESULTS

Irradiation of leukaemia cells in mice breathing 95 per cent air or 95 per cent oxygen

The log survival ratio among the liver leukaemia cells was determined for mice breathing oxygen containing 5 per cent carbon dioxide during irradiation with 800, 1400 or 2000 r total-body radiation   At each dose level the log survival ratio was similarly determined for the leukaemia cells irradiated in a mouse breathing air containing 5 per cent carbon dioxide   The results are recorded in Table I   In Fig 1, the result of each experiment has been entered in the graph relating log survival ratio and radiation dose   The points obtained are seen in relation to the log survival curve previously obtained for the leukaemia cells irradiated in mice breathing air alone (Hewitt and Wilson, 1959)   None of the points departs significantly from the log survival curve previously obtained, and there is no significant difference at each dose level between the survival ratios obtained for cells irradiated in mice breathing 95 per cent air and for cells irradiated in mice breathing 95 per cent oxygen

TABLE I —*Log Survival Ratios among Leukaemia Cells Irradiated In Vivo in the Livers of Leukaemic Mice Breathing* (a) *95 per cent Oxygen,* (b) *95 per cent Air, during Irradiation*

Dose of radiation (r, ^{60}Co gamma rays)	Log survival ratio	
	Mice breathing oxygen	Mice breathing air
800	3̄ 83	3̄ 72
1400	4̄ 55	4̄ 90
2000	5̄ 20	6̄ 53

Irradiation of leukaemia cells in mice immediately after death

It will be appreciated that in the dead mouse experiments the leukaemia cells are allowed to remain in the livers of the dead mice for a period slightly longer than the length of time occupied by the exposure to radiation, and that during this time they would be expected to be under strictly anaerobic conditions and at a temperature falling gradually from 37° C to room temperature   It was conceivable that a proportion of the cells might lose their viability under these
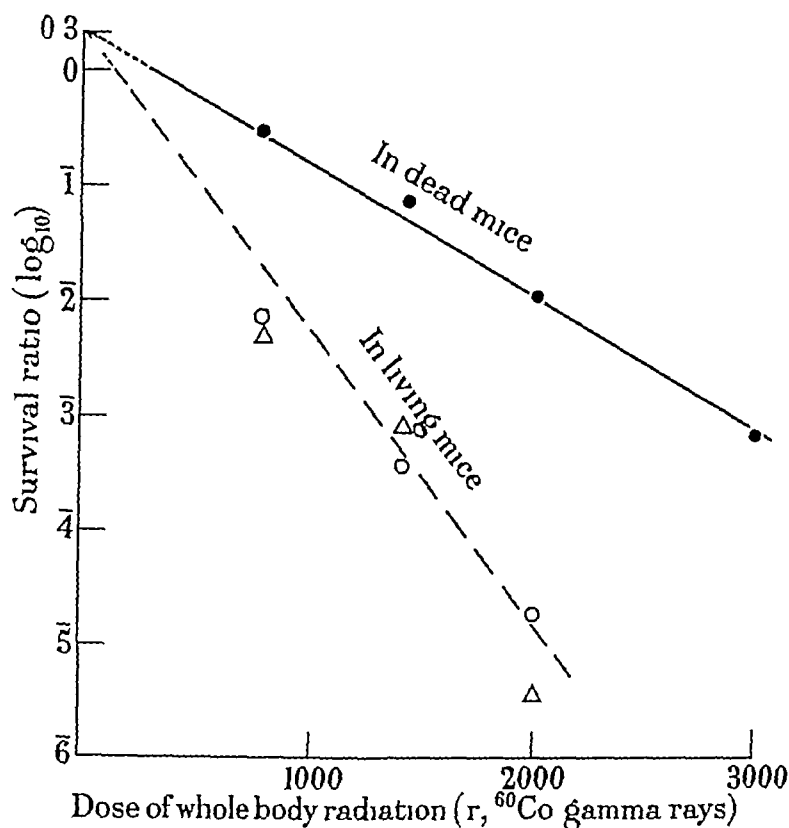
FIG   1 —Survival curves for leukaemia cells irradiated (*a*) in dead mice, (*b*) in living mice

‒ ‒ ‒ ‒ ‒ Survival curve for cells irradiated in living mice breathing air (Hewitt and Wilson, 1959)

O Survival ratios for cells irradiated in mice breathing 5 per cent CO_2 in oxygen
△ Survival ratios for cells irradiated in mice breathing 5 per cent CO_2 in air

conditions   If this were so, the viability loss for cells irradiated in dead mice would be the summation of loss due to anoxia and starvation, and loss due to radiation-induced damage   The longest time of irradiation in these experiments was under 43 minutes (when 3000 r was delivered at a rate of 70 6 r/min)   A preliminary experiment was therefore done in an attempt to detect a rise in the TD50 value given by the leukaemia cells after their residence in the liver of a dead leukaemic mouse at room temperature for 47 minutes after death   A portion of liver was removed from a leukaemic mouse immediately after death by neck fracture and the operation wound was sewn up and the mouse allowed to remain at room temperature   The TD50 of the leukaemia cells released from the excised fragment was then determined   A second liver sample was removed from the mouse 47 minutes after death and the TD50 determined for

the cells in this fragment   The TD50 values obtained were 10 cells and 3 2 cells respectively   It is concluded that the viability loss detected among the cells irradiated in dead mice was due to radiation-induced damage alone and was not contributed to by environmental influences associated with temporary residence of the cells in the tissues of a dead mouse

TABLE II —*Log Survival Ratios Among Leukaemia Cells Irradiated in the Livers of Leukaemic Mice soon after Death*

Dose of radiation (r , ⁶⁰Co gamma rays)	Log survival ratio
800	$\bar{1}$ 44
1400	$\bar{2}$ 83
2000	$\bar{3}$ 98
3000	$\bar{4}$ 79

The log survival ratios obtained for leukaemia cells irradiated in dead mice are recorded in Table II, and it is seen from the upper curve of Fig 1 that there is, again, a linear relationship between log survival ratio and the dose of radiation From the linear part of the curve, which extrapolates to cut the zero dose axis at about +0 3, the D_0 value is approximately 380 r, compared with 165 r for the leukaemia cells irradiated *in vivo* in mice breathing air or oxygen   Thus, for equal survival ratios, the dose required when the cells are under what are assumed to be anoxic conditions is greater than that required when the cells are irradiated in what is assumed to be a moderately well-oxygenated environment, by a factor 2 3 approximately

Theoretical radiation survival curves for leukaemia cell populations consisting of known proportions of anoxic and well oxygenated cells

The linearity of the log survival curves for both anoxic and well-oxygenated leukaemia cells suggests that in each case the cells of the exposed population were remarkably uniform in respect of their environmental oxygen tension   For the cells in dead mice such uniformity is to be expected, since it is inconceivable that foci containing available oxygen could persist among rapidly metabolising cells within an organ whose circulation has ceased   For the cells in mice breathing oxygen or air the apparent uniformity is more surprising   we should expect a proportion of the cells to lie in situations where thrombosis or other vascular accident has given rise to virtually anoxic foci   Areas resembling infarcts in which both the liver cells and the infiltrating leukaemia cells have undergone necrosis, are indeed to be seen occasionally in advanced leukaemic livers   In the case of many solid tumours, which show extensive areas of necrosis in histological section, it cannot be doubted that many of the malignant cells at the boundary of necrotic zones would be under anoxic conditions   Since it appears probable that the cells of many tumours are heterogeneous in respect of the oxygen tension in their environment it is useful to consider the character of theoretical log survival curves for cell populations consisting mostly of well-oxygenated cells but containing a known proportion of anoxic cells, each variety of cell having a radiosensitivity defined by the appropriate D_0 value as determined here *for*

well-oxygenated and anoxic leukaemia cells   It is reasonable to assume that the
respective radiosensitivities would not be influenced by the fact that the cells
belonged to a mixed population, so that the survival ratio for the total population
after any dose of radiation can be expressed as follows

$$\frac{\text{Surviving well-oxygenated cells} + \text{surviving anoxic cells}}{\text{total initial cell population}}$$

With increasing doses of radiation, the viable anoxic cells, being eliminated
at a slower rate than the well-oxygenated cells, will form a rapidly increasing
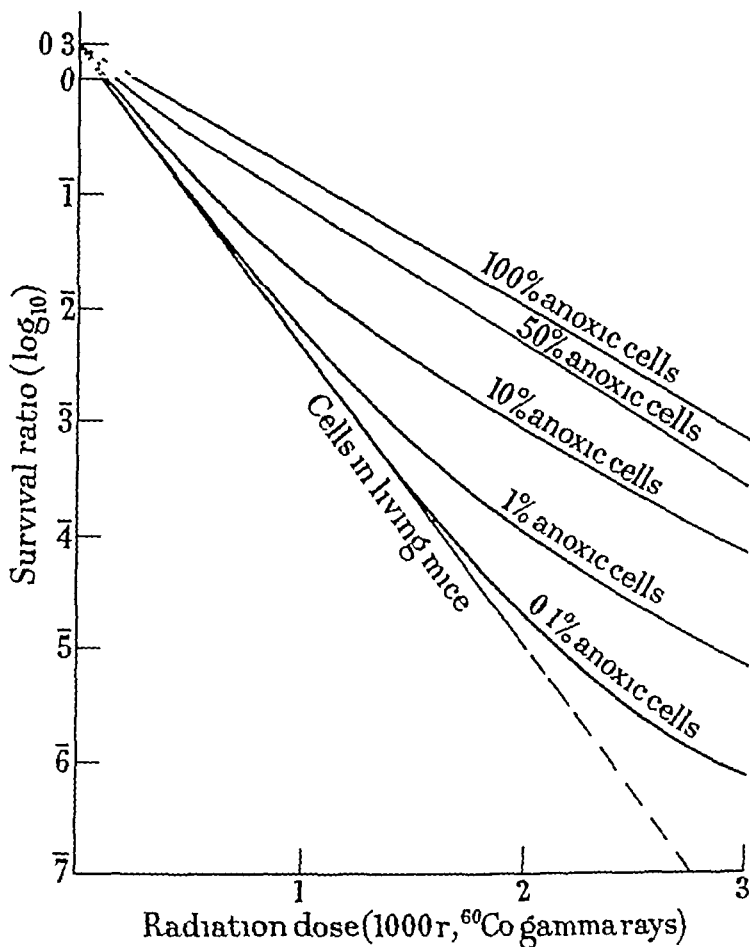
FIG 2 —Theoretical radiation survival curves for leukaemia cell populations
consisting of mixtures of well oxygenated and anoxic cells

proportion of the total surviving population of viable cells   The log survival curve
for the total population will thus gradually assume the slope for a pure population
of anoxic cells   For example, after exposure to 2500 r of an initial mixed popula-
tion of 10^6 cells, consisting of 10 per cent anoxic cells and 90 per cent well-
oxygenated cells, there is less than a 50 per cent chance of one viable well-oxy-
genated cell surviving, whereas about 250 viable anoxic cells would still remain
In Fig 2 the original separate linear log survival curves for pure populations of

well-oxygenated and anoxic leukaemia cells, respectively, are shown Between these, are shown theoretical curves for mixed populations containing various stated proportions of the two types of cell It will be seen that populations containing only a small proportion of anoxic cells give a log survival curve slope which does not depart significantly from that for well-oxygenated cells until higher doses of radiation are attained, when the slope changes gradually to that for anoxic cells

DISCUSSION

A positive correlation between radiosensitivity and environmental oxygen tension has been demonstrated for a wide variety of cells, including several mammalian tumours (Gray, 1957) , the observed ratio of the radiosensitivities of anoxic and moderately well-oxygenated normal tissue cells has been similar to that recorded here for leukaemia cells For example, Howard-Flanders and Wright (1957), using a quite different indicator—the inhibitory effect on bone growth in the mouse tail, found relative radiosensitivity values of 1, 1 97 and 2 56 respectively for the anoxic tail (with occluded blood supply) and the tail in air-breathing and in oxygen-breathing mice Using visible chromosome damage as an index of radiosensitivity for Ehrlich ascites tumour cells irradiated with X-rays at 18° C in vitro, Deschner and Gray (1959) showed that the relative radiosensitivity of the cells rose rapidly from a minimum value of 1 0 for anoxic cells to about 2 3 for cells in fluid equilibrated with oxygen at a pressure of 20 mm Hg With oxygen tensions above this level, radiosensitivity increased more gradually, a value of 3 0 not being attained until the oxygen pressure reached about 400 mm There is no reason to believe that a similar relationship between oxygen tension and radiosensitivity does not obtain for mammalian tumour cells irradiated in vivo, although the environmental oxygen tension of tumour cells in vivo would not be expected to be uniform and would not be measurable with the precision possible with an in vitro system Nevertheless, the results with mouse ascites cells (Deschner and Gray, 1959) and other results with bacteria (Alper and Howard-Flanders, 1956) make it probable that the range of oxygen tensions over which we should expect major alteration of the radiosensitivity of mouse leukaemia cells in vivo is from zero to about 20 mm Hg The range with which we are concerned thus lies distinctly below the tension (40 mm) normally found in the veins of an air-breathing mammal

The local tissue oxygen tension for any small group of tumour cells in vivo cannot at present be ascertained by direct measurement, although it is possible to calculate theoretical values from various assumptions and data Such values have been calculated (Thomlinson and Gray, 1955) for foci within squamous carcinomas of human lung, and have been strikingly correlated with the actual spatial relationships of necrotic foci and capillaries as seen in histological sections of these tumours The complexity of the factors influencing the oxygen tension in the vicinity of tumour cells in vivo, and the effects of raising the partial pressure of oxygen respired have been discussed in great detail by Churchill-Davidson, Sanger and Thomlinson (1957) These considerations cannot, however, provide a reliable assessment of the proportion of viable tumour cells which are actually anoxic in man or animals breathing oxygen at normal or supranormal pressures The theoretical curves shown in Fig 2 suggest that the proportion of mouse

leukaemia cells in the livers of leukaemic mice breathing air or oxygen which are anoxic, is certainly less than 1 per cent and possibly no greater than 0 1 per cent The proportion of anoxic cells in other tumours, those showing more widespread vascular disturbance, may very well prove to be greater

It is clear that the relative radioresistance of anoxic tumour cells is such that the presence of these cells in a tumour *in vivo* would be expected substantially to diminish the effectiveness of tumour radiotherapy   It is, therefore, important to discuss certain theoretical considerations concerning the possible incidence of anoxic tumour cells *in vivo*   It is certain that vascular occlusion frequently leads to death of cells, often involving quite large volumes of tumour tissue Such large-volume necrosis supposedly results from total deprivation of the metabolic requirements of the cells, including glucose, amino acids, vitamins and other growth requirements, as well as oxygen , the accumulation of waste products also may contribute to the necrosis   The predicament of such grossly deprived cells is sooner or later lethal, and their temporary survival in a tumour would have no influence on its radiocurability   The cells whose relative radioresistance would be of importance to radiocurability are those which are almost or actually anoxic but which nevertheless have their viability preserved over the period of time required for them to reproduce over several generations   This situation implies a differential interference with cell requirements, such that adequate amounts of glucose and other growth factors continue to be supplied, while available oxygen falls very severely   We do not know whether the tissue fluid commonly attains a composition which permits these conditions to prevail, and it is clear that more information is required before it can be assumed that groups of tumour cells may pass through a fairly prolonged period of severe hypoxia *in vivo* and later assert themselves as the progenitors of a massive tumour cell population   Our results suggest that such anoxic cells are uncommon, even in the heavily infiltrated leukaemic mouse liver, where anoxic conditions might be expected

Although our results suggest that anoxic cells are unlikely to form more than a small proportion of the total malignant cell population in an air- or oxygen-breathing mouse, it should be appreciated that even a very small proportion of such cells could very significantly affect the dose of radiation required to eliminate the growth potential of a large population of tumour cells   A tumour 2 cm in diameter and consisting half of stroma and half of tumour cells (mean diameter 12 6 μ), with only half the tumour cells capable of reproduction, would contain about 10^9 reproductively intact malignant cells   Now if each of these cells is capable of regenerating a fresh tumour, a survival ratio of about 10^{-10} is required for a 90 per cent chance of eliminating the total malignant cell population   In Fig 3, the linear survival curves obtained for leukaemia cells irradiated under well-oxygenated and anoxic conditions, respectively, have been extrapolated to very low survival rates   It will be seen that a survival ratio of 10^{-10} would be expected after exposure of the malignant cell population to a ^{60}Co gamma radiation dose of 4000 r, provided that all the cells were under well-oxygenated conditions at the time of irradiation   If, however, 10^5 (0 01 per cent) of the cells were under anoxic conditions during exposure, the curve for anoxic cells indicates that after exposure to 4000 r there would be about a 90 per cent chance of one or more reproductively intact cells surviving the irradiation 10^5 cells, of the size given, occupy a volume of only 0 1 c mm   This very small volume of anoxic

cells, present in a tumour of 2 cm diameter, would thus seriously militate against eradication of the tumour by this dose of radiation, and be responsible for reducing an expected 90 per cent cure rate to about 10 per cent   If all the tumour cells were anoxic during irradiation, as would be the case if the vascular supply of the tumour were to be totally interrupted by pressure or torsion, then about 9000 r would have to be delivered to the tumour before a useful cure rate could be expected

The D_0 value obtained by Puck and Marcus (1956) for human HeLa cells irradiated *in vitro* under well-oxygenated conditions with 230 kV X-rays, was 96 r   Puck, Morkovin, Marcus and Cieciura (1957) found a similar value for
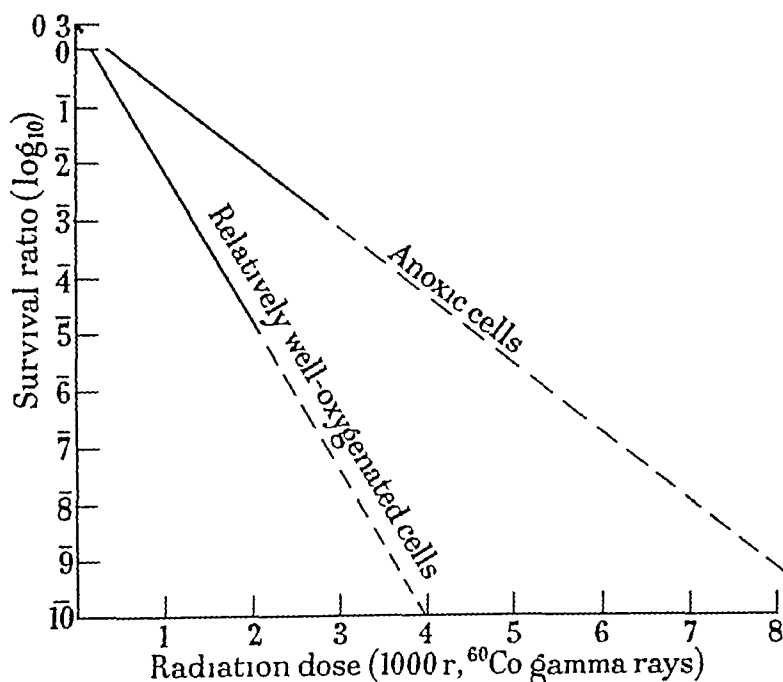
FIG 3 —Extrapolated survival curves for leukaemia cells irradiated under well oxygenated conditions (in air-breathing mice), and under anoxic conditions (in dead mice)

numerous other human epithelial cell types under similar conditions   Recently Morkovin, and Feldman (1959) pointed out that an error in the original dosimetry requires the value of 96 r to be increased by a factor 1 45 to give an adjusted D_0 value of 139 rads   When further increased by the factor 1 25 to allow for the greater R B E of 230 kV X-rays compared with ^{60}Co gamma rays, the D_0 value for human epithelial cells irradiated under well-oxygenated conditions becomes 174 rads of ^{60}Co gamma radiation, which is not significantly different from the D_0 value (161 rads) obtained here for murine leukaemia cells irradiated *in vivo* in air-breathing mice   This remarkably good correlation between the radio-sensitivities of human and murine cells suggests that parameters obtained from radio-biological studies of mouse tumours may be directly applicable within the sphere of clinical radiotherapy of tumours   It may be added that consideration of the implications of such parameters should properly precede the use of such procedures as the treatment of human leukaemia by whole-body radiation

SUMMARY

A transplantation bio-assay method was used to determine survival ratios among the leukaemia cells released from the livers of leukaemic mice immediately after their exposure to 800, 1400 and 2000 r total-body ^{60}Co gamma radiation, a surviving cell being defined as one capable of securing successful transplantation of the leukaemia No significant difference was demonstrated between the survival ratios obtained for cells from mice breathing 95 per cent oxygen and from mice breathing 95 per cent air during irradiation None of the survival ratios departed significantly from the linear log survival ratio-radiation dose curve obtained previously for leukaemia cells irradiated in mice breathing air (Hewitt and Wilson, 1959) A linear relationship was demonstrated between log survival ratio and radiation dose for the leukaemia cells irradiated under anoxic conditions (in recently killed mice) Comparison of the log survival curves for cells irradiated in mice breathing air or oxygen and for the anoxic cells showed that the latter were more radioresistant by a factor 2 3 The slope of the log survival curve for cells irradiated in living mice was closely similar to that obtained by Puck and Marcus (1956) for human cancer cells (HeLa) irradiated under well-oxygenated conditions in vitro

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QUANTITATIVE STUDIES WITH MH2 RETICULO ENDOTHELIOMA VIRUS

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THE chorioallantoic membrane (CAM) of a developing chick embryo has been extensively used for the study of the quantitative aspects of the multiplication of Rous Sarcoma virus Rous and Murphy (1912) first demonstrated that when the Rous Sarcoma I virus was inoculated into the embryonic membranes of a chick embryo tumours developed at the site of inoculation Keogh (1938) inoculated Rous Sarcoma virus extract on the intact ectodermal surface of the CAM and produced ectodermal lesions or pocks The quantitative aspects of titrating Rous Sarcoma virus extracts on CAM has been followed up by Rubin (1955), Prince (1958a, b, c) and Vigier (1959) Unlike Keogh, these workers did not experience the large range of variation between eggs which had led to the neglect of assaying these viruses on CAM

The present study deals with some aspects of the quantitative titration of MH2 reticulo endothelioma virus (Murray and Begg, 1930), especially on the CAM.

MATERIALS AND METHODS

Preparation of virus extract

Crude extracts were prepared by grinding tumours from chicks or infected CAMs in 10 volumes of sterile water in glass homogenisers The cells and debris were removed by centrifuging at 1000 g for 10 minutes

Purified preparations of virus according to the method of Bather (1953), were used in a few of the parallel titrations

Titration of virus

Embryos and day old chicks used for titration were obtained from the inbred flock of Brown Leghorns selected for susceptibility to Rous sarcoma virus by Dr Carr and maintained at the Poultry Research Centre

The extracts were titrated on the CAM of chick embryos according to the method of Keogh (1938) Embryos of various ages (6–12 days old) were used A triangular window was drilled very carefully to minimize damage to the CAM and the CAM was dropped in the usual manner 0 1 c c of extract was inoculated on the dropped CAM and the window was sealed with Scotch tape The embryos were then incubated at 38° C for 7 days The eggs were candled daily to detect dead embryos To count the pocks, the CAMs were carefully cut out, rinsed in water and spread out on a petri dish which had a grid marked out on its back. The pocks were counted against a dark surface with a tally counter

Titrations in day old chicks were done according to the method of Carr and Harris (1951) 0 2 c c of ten-fold dilutions were inoculated into the right thigh in groups of 3 or 4 day old chicks The virus titre was calculated according to the method of Parker and Rivers (1936)

RESULTS

Percentage of non reactors

The percentage of non reactors to Rous Sarcoma virus was used by Prince (1958*a*) to select the most susceptible strain of embryos for the CAM assay technique He found that White Leghorns produced the least percentage of non reactors, only 10 per cent of embryos failing to produce any pocks even when inoculated with very high doses of virus Vigier (1959) also found non reactors but their number decreased with increase of virus titre

Table I gives the percentage of non reactors for the different ages of embryos of Brown Leghorns used To make the results comparable with those of Prince the percentage of non reacting membranes is only calculated from groups of embryos having an average of at least 20 pocks so that distributional zeros may not be included

TABLE I —*Relationship Between Percentage of Non Reactors and the Age of Embryos Used*

Age of embryos (days of incubation at 38° C)	Number of CAMs tested	Percentage of non reactors
6	44	11 3
7	40	5 0
8	91	5 5
9	63	1 6
10	195	0 5
11	20	0
12	38	0

From Table I it is obvious that the percentage of non reactors is reduced with increasing age of embryos This would support the hypothesis that non reactors are not due to the presence of neutralising antibody in the blood stream of the embryos (Prince, 1958*b*) It is known that maternal antibody is transmitted to the embryo through the ovary via the yolk (Andrewes, 1939) It has been shown that antibody present in the yolk is released into the circulation of the embryo in greater amounts with increasing age of embryo (Schechtman and Knight, 1955) Thus, if neutralising antibody was the cause of non reactors it would be expected that the percentage of non reactors would increase with the age of embryos

Effect of age of embryo on pock count

To compare the number of pocks produced by embryos of different ages standard amounts of the same extract were inoculated into groups of embryos of different ages Eight to twelve embryos were usually inoculated for each age group Embryos of four different ages, viz 6, 8, 10 and 12 days old were used for these experiments

TABLE II —*Mean Number of Pocks Produced on CAMs After Inoculation of Same Dose of MH2 Virus Extract on Chick Embryos of Different Ages*

	Age of embryos					
	6 days			8 days		
Expt No	Number of embryos	Mean ± SE	Coeff of var	Number of embryos	Mean ± SE	Coeff of var
I	9	18 3±6 3	96 3	11	21 7±7 5	114 6
II	4	26 3	—	4	28 7	—
III	6	5 5±1 2	43 3	8	8 7±4 2	135 5
IV	—	—	—	8	6 7±1 7	74 1
V	—	—	—	12	17 7±4 9	95 9
VI	—	—	—	8	12 4±2 4	54 6

	10 days			12 days		
Expt No	Number of embryos	Mean ± SE	Coeff of var	Number of embryos	Mean ± SE	Coeff of var
I	12	43 3±5 2	41 1	11	48 9±7 3	49 4
II	6	46 5	—	8	58 6	—
III	8	63 2±7 5	35 3	8	58 2±9 7	40 9
IV	8	32 8±4 5	30 8	—	—	—
V	9	31 5±6 5	58 6	—	—	—
VI	8	42 4±7 1	44 1	—	—	—

The results of the various age experiments are summarized in Table II Although there is a slight overall increase in the number of pocks associated with an increase in the age of embryos (the only exception being Experiment III where there is a slight decrease in the number of pocks from 10 day to 12 day embryos) the greatest difference is between 8 day and 10 day old embryos There is some variation between experiments as to the increase in the number of pocks between embryos of these two age groups, but from the means for all the experiments it appears that on the average 10 day old embryos produce 2½ times as many pocks as 8 day old embryos It was also noted that the pocks in 12 day and 10 day old embryos were slightly larger than those in 8 day and 6 day old embryos However, there was a large range of variation in the size of pocks within each group

Comparison between virus titre values from day old chicks and pock counts on CAM

Parallel titrations in day old chicks and on the CAMs of mostly 10 day old embryos are compared graphically in Fig 1 and summarized in Tables III, IV and V The details for the various experiments are given below As seen in Fig 1 and Tables III, IV and V, the titration values from day old chicks are much higher than the corresponding values from the pock titre on CAM The last column in Tables III, IV and V gives the log difference between the two titration values It appears that the CAM assay method is 1 log to 2 log less sensitive than the titrations in day old chicks

Table III gives the results of three experiments where the virus titres were compared without passage on the CAM (1st transfer generation), and after one passage to see if there was any adaptation In all the experiments there appears
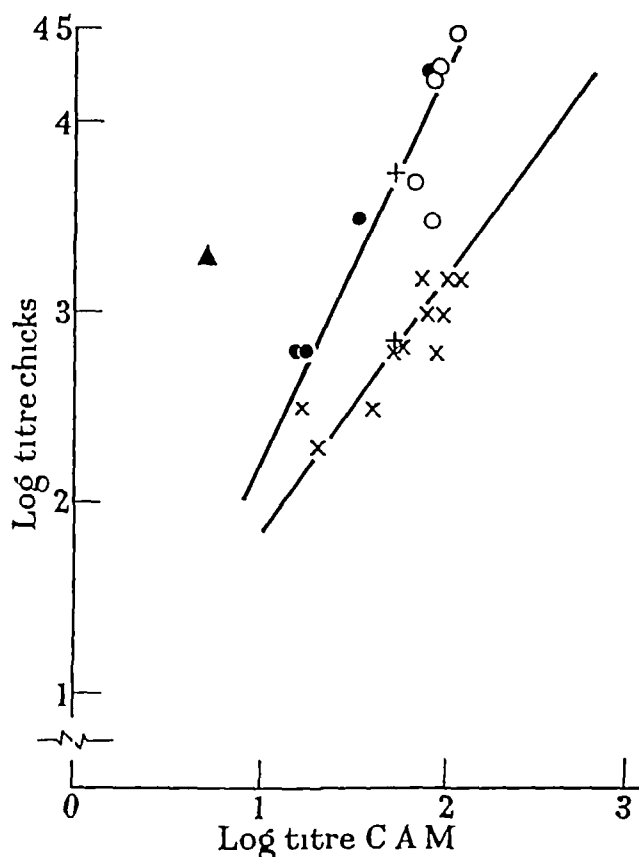
Fig 1 —Relationship between titres calculated from pocks on CAM
and the end point technique in day old chicks

Regression (b) for chick derived virus = 0 45
Regression (b) for CAM derived virus = 0 72

(1) Difference between the two regression coefficients is not significant (0 5 > P > 0 1)
(2) Sample difference in elevation of the two regression lines is highly significant (0 001 > P)

Mean of each group indicated by +

● 1st transfer to CAM
× Further passages on CAM
○ Multiple CAM passages followed by 1 or 2 in chicks
▲ 8 day embryos used

TABLE III —*Relationship Between Titration Values from Pock Counts on CAM
and Day Old Chicks Without Passage on CAM and After One Passage*

Expt No	Transfer generation on CAM	Mean pock count CAM 0 1 c c	Mean pock count CAM 0 2 c c	Log pock titre CAM 0 2 c c	Log virus titre chicks 0 2 c c	Log difference
I	1	7 25±0 9	14 5	1 16	2 8	1 64
	2	51 12±7 5	102 24	2 01	3 2	1 19
II	1	2 4 ±0 06	4 8	0 61	3 2	2 62*
	2	7 84±0 54	15 68	1 2	2 5	1 3
III	1	15 77±1 84	31 54	1 5	3 5	2 0
	2	34 71±2 39	69 42	1 84	3 2	1 36

* Use of 8 day-old embryos

Note   1st transfer generation on CAM = virus derived from chick tumour without any previous
passage on CAM

TABLE IV —*Relationship Between Titration Values from Pock Counts on CAM and Day Old Chicks After Passage of Virus on CAM for a number of Transfer Generations*

Expt No	Transfer generation on CAM	Mean pock count CAM 0 1 c c	Mean pock count CAM 0 2 c c	Log pock titre CAM	Log virus titre chicks	Log difference
I	1	9 57±0 97	19 14	1 28	2 8	1 52
	2	47 25±4 28	94 5	1 98	3 2	1 22
	3	42 0 ±5 69	84 0	1 92	3 0	1 08
	4	18 83±2 61	37 66	1 57	2 5	0 92
	5	24 25±1 92	48 5	1 69	2 8	1 11
II	1	36 2 ±4 93	72 4	1 86	4 3	2 44
	4	40 25±2 69	81 7	1 91	2 8	0 89
	5	37 5	75 0	1 88	3 0	1 12
	6	6 71±1 66	13 42	1 28	2 3	1 02
	7	24 5 ±1 75	49 0	1 69	2 8	1 11

Note 1st transfer generation on CAM = virus derived from chick tumour without any previous passage on CAM

TABLE V —*Relationship Between Titration Values from Pock Counts on CAM and Day Old Chicks After a Number of Passages on CAM Followed by a Number of Passages on Chicks*

Details of transfer generation (tg)	Mean pock count CAM 0 1 c c	Mean pock count CAM 0 2 c c	Log pock titre CAM	Log virus titre chicks	Log difference
5 tg on CAM and 1 tg on chicks	42 4±7 1	84 8	1 93	3 5	1 57
7 tg on CAM and 2 tg on chicks	31 3±5 87	63 6	1 80	3 7	1 9
11 tg on CAM and 1 tg on chicks	57 8±5 68	115 6	2 06	4 5	2 44
7 tg on CAM and 4 tg on chicks	36 0±4 52	72 0	1 86	4 3	2 44
7 tg on CAM and 5 tg on chicks	35 7±3 28	71 4	1 85	4 3	2 45

to be a slight adaptation to the CAM after one passage—the log difference between the two titration values being significantly less after one passage on the CAM In Experiment II in the 1st generation on CAM there is a greater difference between the two values than in the other experiments due to the use of 8 day old embryos for titration on CAM

Table IV gives a comparison between titration values after the virus had been propagated for a large number of transfer generations on the CAM to see if any further adaptation would take place The first transfer generation on CAM indicates that the virus is passed on the CAM for the first time Continual passage of virus on the CAM does not make any difference to the extent of adaptation, the difference remaining about 1 log

To see if the adaptation to the CAM is permanent, virus, after a number of transfer generations on CAM, was inoculated into chicks and passed for one or more transfer generations on chicks Parallel titrations were then carried out on CAM and day old chicks The results and details of transfer generations are shown in Table V In Table V the log differences in virus titre after even one passage on chicks are comparable with the log difference shown in Tables III and

IV when the virus had not been passed on the CAM at all The overlapping of these points can be seen in Figure 1 From this it appears that the slight adaptation obtained after one or more passages on CAM is lost as soon as the virus is returned to chicks

The difference in log titre when the virus is titrated on the CAM for the first time varies from 1 5 log to as much as 2 5 log in some of the experiments After one or more passages on CAM, i e after adaptation, this difference remains fairly constant around 1 log

DISCUSSION

The titration of MH2 reticuloendothelioma virus on CAM of chick embryos shows a number of interesting results The percentage of non reactors in the strain of Brown Leghorns used, after 9 to 12 days of incubation, is well below that obtained by Prince (1958a, b) using 12 day old embryos even with the most susceptible strain (White Leghorns) Prince (1958b) also showed that the difference between the highly resistant strain (Fayoumi) and the highly susceptible strain (White Leghorns) can be explained on a genetic basis He found that resistance was a genetic trait which was neither sex-linked nor passed by maternal inheritance His data agreed with the hypothesis that resistance was controlled by a single pair of allelic genes, the allele for sensitivity being dominant to that of resistance

The extremely low percentage of non reactors obtained can be explained by the fact that the embryos used are derived from a line of birds selected for susceptibility to Rous sarcoma virus over a number of generations Susceptibility to one type of tumour virus may result in susceptibility to other types of tumour viruses

The reason for the large increase in the number of pocks between 8 day and 10 day old embryos is unknown On about the 9th day of incubation the haematopoietic tissue in the peripheral blood of embryos reaches stability (Fennell, 1947) The mean percentage of primitive erythroblasts decreases while that of definitive erythrocytes increases to over 90 per cent from about 10 per cent on the 7th day of incubation Between 9 and 12 days of incubation Fennell (1947) observed the appearance of thrombocytes in the peripheral circulation of chick embryos The most striking change after the 10th day is the extensive vascularisation of the CAM From the 10th day the capillaries begin to push outwards towards the shell membrane so that by the 15th day the CAM is extensively covered by a fine network of capillaries with a few ectodermal cells reaching the surface through the capillary net (Danchakoff, 1917) The increased number of pocks between 8th and 10th day embryos may be associated with the various haematopoietic changes in the peripheral circulation of the embryo It may be due to the extensive vascularization of the CAM especially as the MH2 is a reticuloendothelioma virus and would tend to infect the walls of blood vessels The increase in pocks may be connected with the biochemical requirements of the virus correlated with the coming into function of some essential organ in the chick embryo, e g between 9 to 12 days the spleen begins to act as a haematopoietic organ (Olson, 1943) It is possible that only variants lacking certain biochemical requirements are able to grow in younger chick embryos

The CAM assay method has certain advantages over the other titration techniques available for avian tumour viruses While all the other titration methods are based on end point techniques in fowls and chicks (Claude and Rothen, 1940 ,

Carr and Harris, 1951 , Bryan, 1955) the CAM titration gives a direct count of the number of infective virus particles Moreover, the CAM titration gives results in a much shorter period compared with the other techniques

The difference in the values obtained by titrating in day old chicks and by the pock count technique on CAM is very striking for the MH2 virus Rubin (1955) using Rous sarcoma virus compared the titre obtained by the pock count technique on the CAM with those obtained by the subcutaneous inoculation of 2 weeks and 8 weeks old chicks He found the CAM technique to be more sensitive than the 50 per cent end point technique on birds However, when he compared his results with those of Bryan (unpublished), who assayed the same stable stock of virus, he found that the CAM assay agreed very closely with the average of the ID 50 assays in chickens Prince (1958a) also compared the titres obtained by the CAM technique with those obtained by the wing web assay technique for the same virus He found no difference between the two titration values Vigier (1959) also found no difference between pock titre from CAM assay and the end point titre in 1–2 months old chicks for Rous sarcoma virus

The difference in the titration values by the two methods for the MH2 virus is less likely to be due to virus variants Compared with the Rous sarcoma virus, a virulent strain, which has been passaged almost continuously for 50 years, the MH2 is of more recent isolation, has been passaged much less (much of its existence being spent as a freeze-dried conserve) and is very much less virulent in older chicks (Carr, personal communication) It is possible that the increased suscepti-bility of the day old chicks as against the CAM is a continuation of the age effect The CAM is a short-lived tissue with a life-span of about 19 days and in 10 day old embryos the CAM is already an ageing tissue and hence might be less sensitive to the MH2 virus On the other hand, the embryo itself is more sensitive to MH2 virus than the day old chick for intravenous titrations of MH2 virus in groups of 14 day old embryos gave an end point which was much higher than that obtained by titrating the same extract in day old chicks (unpublished data)

Passage of the virus on the CAM for one generation produces a slight adapta-tion to the CAM which is lost as soon as the virus is returned to chicks The fact that the difference in log titre is more variable before adaptation and becomes more constant after, suggests that the change is due to a selection of a variant It may be a variant with increased capacity to infect uninjured cells or the adaptation may be due to a selection of an ectodermal specific variant which would be lost as soon as the virus is injected into the muscle of the chick

It is clear that the results obtained with the Rous sarcoma virus are not applicable to other fowl tumour viruses and that the quantitative techniques evolved for this virus are, in the absence of any other information, best restricted to that virus

SUMMARY

When MH2 virus is assayed on the chorioallantoic membrane the result depends very largely on the age of the embryo, the pock count on membranes of embryos aged 10 days being $2\frac{1}{2}$ times that on those 8 days old This was always much less than that determined in the same strain of day old chicks by the limiting dilution method Slight adaptation of the virus to egg passage was found but this was lost when the virus was transmitted through chicks Non reactor eggs were

few, and the number decreased markedly with the increasing embryo age, to vanish at age 11 days and over

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INVESTIGATIONS OF A FACTOR FOUND IN CERTAIN NORMAL TISSUES INHIBITING ASCITES TUMOR GROWTH IN THE RAT AND MOUSE

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IT has long been known to pathologists that apparently normal cancer cells may circulate in the blood stream of cancer patients and eventually lodge in the lungs or other tissues where the great majority are inactivated and do not cause metastases   This may be merely a question of viability and the stroma-inducing ability of the cancer cells, but there is also a possibility that antibodies, or some local intrinsic tissue factor might be responsible   The last possibility has been under investigation by Druckey et al for several years (Druckrey, Schmahl and Rajewsky, 1958 , Steinhoff, Flaschentrager and Bannasch, 1958 , Schmahl, Bannasch and Flaschentrager, 1958)   Druckrey uses a simplified experimental system, consisting simply of incubating Yoshida ascites cells for several hours with various normal rat tissue homogenates, and then injecting the incubated cells intraperitoneally into rats of the same strain   Druckrey found that several tissue homogenates were responsible for a marked inhibition of tumor growth on reinjection (Druckrey, Schmahl and Rajewsky, 1958) and for microscopic damage to the tumor cells (Steinhoff, Flaschentrager and Bannasch, 1958)   The effect was especially prominent with lung and spleen homogenates

The experiments to be described were designed to elucidate further the nature of this phenomenon and the characteristics of the factor responsible   It was attempted

(*a*) to verify Druckrey's results on other tumors and species,

(*b*) to eliminate by a series of checks and controls the possibility that the effect was due to physical or chemical properties of the tissue homogenates (pH, osmolarity, electrolyte composition) ,

(*c*) to see whether a tumor normally metastasizing to the lung would still be affected similarly by lung tissue ,

(*d*) to see whether tissue homogenates from tumor-bearing animals act in the same way as those from healthy animals ,

(*e*) to determine which cell fraction or fractions contain the responsible factor

MATERIALS AND METHODS

Species and tumors

(1) The " G–6 " tumor in Wistar WAG pure bred white rats   This was originally a spontaneous mammary tumor of the rat , in its solid transplantable form it metastasizes frequently to the lungs   The ascitic form used here had been passed in the Wistar rat for 30 generations and had attained a uniform pattern,

5×10^6 cells injected intraperitoneally regularly killing the animal, without metastases, in 12 to 15 days

(2) The Ehrlich ascites tumor, in pure bred white mice of Strain A   Intraperitoneal injection of 5×10^6 cells caused the animal's death in about 20 days

Preparation of tumor cells

Ascites fluid was removed from a tumor-bearing animal, and centrifuged slowly (2000 g) for 10 minutes to sediment the cells   The cells were then resuspended in their own volume of saline   This solution was used for the incubations, within 30 minutes of withdrawal from the animal

Preparation of tissue extracts

The same procedure was used for rats and mice, the animals providing the tissue always being of the same pure strain as those bearing the tumors

Saline was perfused through the still beating heart to rid the animal's tissues of blood in so far as possible   The tissues to be used were removed, mixed with an equal weight of saline, homogenized for 30 seconds at 0° C in an Ultra-turrax homogenizer (20,000 r p m) and then centrifuged at 2500 g for 15 minutes   The supernate was used for the incubations

In one section the lung homogenate was boiled for five minutes before centrifugation, to make the " protein-free " lung extract

In the cell-fraction experiments the tissue was homogenized in a Potter homogenizer (1000 r p m) for 5 minutes, instead of the Ultra-turrax to avoid damaging nuclei   The solution was strained to remove large particles and then centrifuged in the Spinco " 40 rotor " at 40,000 r p m (150,000 g) for 30 minutes   Both the supernate and sediment were used in incubations, the sediment, either whole or rehomogenized for 30 seconds in the Ultra-turrax, being resuspended in saline, 1 c c for 0 2 g tissue

Incubation

The tissue extracts or controls prepared as described were then incubated with the tumor cell solutions for 2 hours at 37° with agitation, in a ratio of 3 volumes extract to one volume of ascites cell solution   At the end of the incubation the mixture was immediately injected intraperitoneally into fresh animals of the same strain, generally 5×10^6 cells per animal

In some cases, after 2, 3, or 4 hours of incubation, smears of each tube were made and stained by the May–Gruenwald Giesma technique for microscopic examination

In the *in vivo* experiments the extract of lung tissue, prepared as above, was injected intraperitoneally several days after the tumor cells, without incubation

RESULTS

pH   The pH was between 6 5 and 7 0 in all incubation tubes

Microscopic appearance

No striking cytolytic effects such as described by Druckrey for the Yoshida tumor (Steinhoff, Flaschentrager, and Bannasch, 1958) were noted with either

of the tumors used here   There was a tendency for the tumor cells incubated with lung or spleen extracts to show more nuclear pyknosis, more cell shrinkage and more cell membrane rupture than the controls   Unfortunately, however, the microscopic appearance could not always be used as a reliable guide to the viability of the cells on reinjection, in either the G–6 or the Ehrlich ascites tumor

Results on reinjection—G–6 tumor

(1) *Whole-tissue extracts (Tables I, II, III)* —It was found that the extracts prepared from lung or spleen had a definite inhibitive effect on the growth of this ascites tumor ($p < 0 01$)   The extract from liver (Table III) was without effect Extract made from the lungs of animals bearing the G–6 tumor was as effective as that made from lungs of healthy animals (Table II)   The " protein-free " lung extract had no inhibitive effect (Table II)

TABLE I —*Development of Ascites in Rats Injected Intraperitoneally with G–6 Ascites Tumour Cells Variously Treated*

	Group		
	1	2	3
Ascite cells incubated with	Saline	Whole lung extract	Whole spleen extract
Rat 1	385	0	0
2	528	0	0
3	300	0	0
4	396	2	0
5	432	300	0

The numbers represent the total number of ascites cells (in millions) found in each of the fifteen animals, all killed 20 days after tumor inoculation   i e   Quantity of ascitic fluid (c c) times cell count (per c c) times 10^{-6}

TABLE II —*Development of Ascites in Rats Injected Intraperitoneally with G–6 Ascites Tumour Cells Variously Treated*

	Group				
	1	2	3	4	5
Ascites cells incubated with	Saline	PFLE	WLE	WSE	WLE T
Rat 1	528	468	3	100	0
2	555	648	0	190	0
3	270	288	0	150	0
4	0	240	0	756	0

The numbers represent the total number of ascites cells (millions) found in each of the twenty animals, all killed 15 days after tumor inoculation   PFLE   protein free lung extract, i e   lung extract heated to 100° C for five minutes before centrifugation   WLE   whole lung extract   WSE whole spleen extract   WLE T   whole lung extract made from lungs of rats bearing the ascites tumor

(2) *In vivo injection* —Injection of the whole lung extracts into rats 3 and again 5 days after ascites cell injection resulted in a slight but so far not significant inhibition in tumor growth   These intraperitoneal injections were not toxic to the animals systemically nor was there any evidence of local damage

(3) *Cell fraction studies* —Here incubation of G–6 cells was carried out with different ultracentrifuge fractions of rat lung tissue   The results of one such

TABLE III —*Survival Times After Inoculation with G-6 Ascites Tumor Cells Variously Treated*

Cells incubated with	1 Saline	2 Liver E	3 SLE	4 Sed LE	5 Sed LERH
Rat 1	15	16	15	20	37
2	15	17	16	23	45+
3	16	17	17	23	45+
4	17	18	17	21*	45+
5	23	19	18	—	45+

Showing survival times in days of 24 rats, after tumor inoculation Groups 1 and 2 form one experiment, groups 1, 3, 4, 5 form another Liver E whole liver extract Has no connection with ultracentrifuge studies SLE supernate of lung extract centrifuged at 150,000g for 30 minutes Sed LE sediment of lung extract Sed LERH sediment of lung extract rehomogenized in the ultraturrax 45+ indicates animal was living and well after 45 days * this animal lost its identification but almost certainly belongs in group 4

experiment are presented in Table III It is seen that the supernate fraction, containing the cytoplasmic proteins (J Chauveau, personal communication) had no inhibitive effect , the " whole sediment " fraction, containing nuclei, mitochondria, and " microsomes " (J Chauveau, personal communication , De Duve et al , 1955) had a slight effect (borderline significance) , the " rehomogenized sediment " fraction, containing broken nuclei and generally undamaged mitochondria and " microsomes ", had a definite significant effect ($p < 0 01$)

Another similar experiment tended to show similar results, but with a definite effect also in the " whole sediment " fraction

Results on reinjection—Ehrlich tumor

From Table IV it is evident that in the Ehrlich ascites tumor likewise, lung and spleen extracts from pure-bred mice of the same strain definitely inhibited the growth of the tumor ($p < 0 01$) Again the effect was not found in " protein-free " lung

TABLE IV —*Survival Times After Inoculation with Ehrlich Ascites Tumor Cells Variously Treated*

Ascites cells incubated with	1 Saline	2 Protein free lung	3 Whole lung	4 Whole spleen
Rat 1	30	24	120+	18*
2	30	27	120+	45*
3	32	28	120+	120+
4	37	30	120+	120+
5	37	—	120+	120+
6	41	—	120+	120+
7	65	—	120+	120+
8	120+			

Showing survival times in days of 28 mice after tumor inoculation 120+ indicates animal was living and well after 120 days * indicates animal died *without* ascites All deaths not so marked occurred with definite ascites on autopsy

In another experiment it was found, surprisingly, that rabbit lung, prepared in the same manner, did not have the inhibitive effect found in the mouse lung

DISCUSSION

From these results, as well as Druckrey's with the Yoshida tumor, it seems reasonable to conclude that there is a factor capable of inhibiting ascites cell growth, at least in the lung and spleen of the species studied

It is to be noted that the rat lung tissue was effective against the G–6 tumor, although this tumor in its normal form readily metastasizes to the lung

Since the protein-free lung extracts were always without effect, the inhibiting factor should be looked for among the proteins   Since the tissue source was always the same animal strain as that bearing the tumor, and since a number of specific immunological trials for reaction between lung tissue and tumor were always negative, we can almost certainly rule out the possibility that the inhibiting factor is an antibody

The results of the ultracentrifuge experiments make it likely that the factor is found in cell nuclei, since the rehomogenized sediment fraction appeared much more active than the whole sediment fraction, from which it differed presumably only in having its nuclei broken and nuclear protein released   It has not yet been possible to do further subfractionation studies due to the amount of tissue needed for inhibition , thus a mitochondrial or microsomal location for the factor is still possible, but the cytoplasmic protein fraction has been ruled out in two experiments

It is interesting that the factor is present in lung and spleen but apparently not at all in liver similarly prepared   (Druckrey found several other tissues also relatively or absolutely lacking in the factor, and claims that serum not only lacks inhibitive power but actually enhances the ascitic growth   Landschutz (1956), however, claims to have noted distinct inhibition of several ascites tumors by human serum   From these differences one might be led to suspect some cellular enzyme found in the lung and spleen but relatively little in the liver   ATPase or Hexokinase would fit these conditions in the rat (Spector, 1956) but it would be rather surprising to find the effect principally in the nuclear fraction if one of these enzymes were responsible

Further work is needed to isolate the factor and test whether other, non-ascitic, tumors could be affected, and whether normal cells are not damaged as well   It may be possible to answer the latter question to some extent in tissue culture   So far no damage to the animal, locally or systemically, has been found, at least with the intraperitoneal injections used

The rough in vivo experiments, which did show some tumor inhibition even with only two tissue injections, timed arbitrarily, may show promise

SUMMARY AND CONCLUSIONS

The effect of a normal tissue factor, previously found to inhibit the growth of Yoshida ascites cells after in vitro incubation, has been investigated on several ascites tumors of the rat and mouse

Such a factor was demonstrated in rat lung and spleen, inhibiting the growth of the G–6 ascites tumor, and in mouse lung and spleen, inhibiting the growth of the Ehrlich ascites tumor   The factor is not found in liver tissue   It is found in lung tissue from animals bearing the tumor under investigation as well as in lung tissue from normal animals   The factor is protein in nature, but is

absent from the lung cytoplasmic protein , it is present most probably in the nuclear fraction   The tissue extracts used are not toxic on intraperitoneal injection and have a slight ascites-inhibiting effect *in vivo*

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SERIAL IRRADIATION OF MOUSE TUMOURS EFFECTS ON TUMOUR ESTABLISHMENT TIME AND GROWTH RATE

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SOME authors have shown that a reduced growth rate occurs in animal tumours after single or repeated irradiations Russ (1924) observed this in post-irradiation tumours to the extent of one-quarter of the control growth rates Sugiura (1934) also observed this effect in recurring Sarcoma 180 tumours after irradiation with a sub-lethal dose Snellman (1935) reported similar findings in the Jensen rat sarcoma subjected to serial irradiations, even after transplantation into fresh animals However, Hill, Morton and Witherbee (1919) found no changes in tumour establishment time or growth rate after serially irradiating Adeno-carcinoma 63 for 14 generations

The present communication reports the effects of serial irradiations on the establishment times and growth rates of Sarcoma 37 and two homologous transplantable mouse tumours

MATERIALS AND METHODS

RIII strain inbred mice were used throughout this study The transplantable tumours, from which the serially irradiated lines were developed, were Sarcoma 37 and two homologous tumours of the RIII strain—a spindle-celled sarcoma (BP1) and a mammary adenocarcinoma (MV212) The establishment of the irradiated lines has been described in a previous communication (Pearson, 1959) Three irradiated lines were treated with sub-lethal doses (Sarcoma 37 " B " line, BP1 " F " line and MV212 " G " line) and the fourth treated with half-lethal doses at each stage (Sarcoma 37 " D " line)

Recurring post-irradiation tumours or those from untreated sub-line passages were used for donor material in the growth rate estimations 1 mm³ portions from the cortex of parent tumours were inoculated subcutaneously into the right flanks of the experimental animals Tumour area was employed in the comparisons and was calculated as the product of the major and minor axes measured by calipers

The growth rates of Sarcoma 37 " B " line tumours were measured at the 12th irradiation stage after 5, 15, and 32 untreated sub-line passages (B12/5, B12/15 and B12/32) and at the 17th stage after 3 sub-line passages (B17/3) Sarcoma 37 " D " line tumours were measured at the 10th irradiation stage after 1 and 8 sub-line passages (D10/1 and D10/8) The growth rates of the " F " and " G " irradiated lines of the homologous spindle-celled sarcoma and mammary adenocarcinoma, were made at the 1st sub-line passage from the 7th

and 9th irradiation stages respectively The numbers of mice employed in each group are shown in brackets in Fig 1–4

RESULTS

The average growth rates of tumour groups from the control and irradiated " B " lines of Sarcoma 37 are shown in Fig 1 The marked increase in tumour establishment time and reduction in growth rate was of the same order for the 5th, 15th and 32nd sub-line passages from the 12th irradiation stage Tumours from the 17th irradiation stage exhibited a further increase in the establishment time Tumours from the 10th irradiation stage of the Sarcoma 37 " D " line
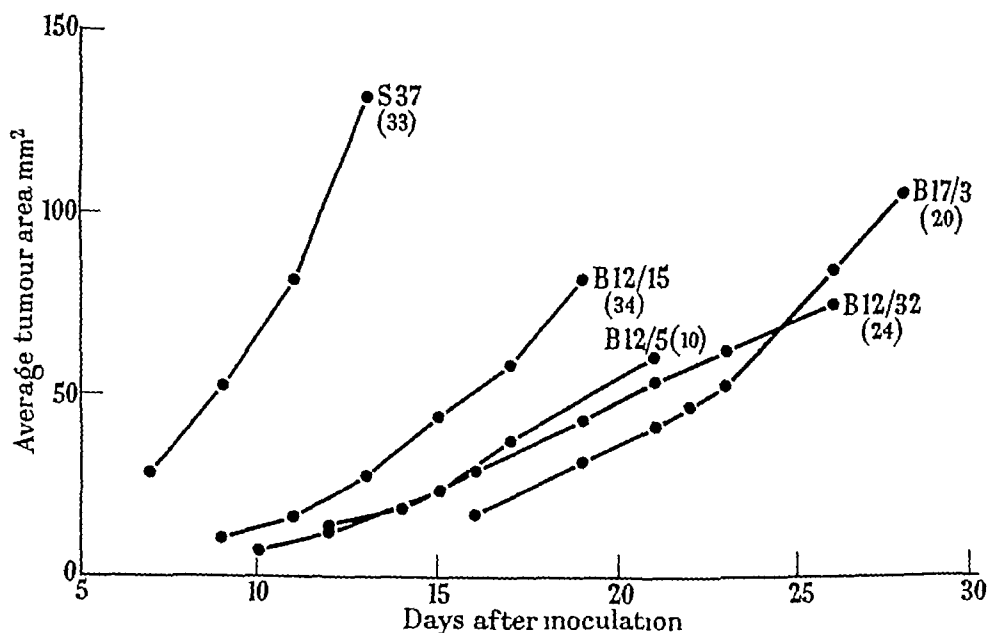
FIG 1 —Growth rates of Sarcoma 37 and its serially irradiated " B " line Figures in parentheses denote numbers of mice employed in each group

showed similar but less marked growth changes (Fig 2) and again sub-line passages without further irradiation did not alter this effect

The increase in establishment time occurred gradually This period (calculated from inoculation to the attainment of about 15 mm ^{2} in area) was 7 days up to the 4th irradiation stage in the " B " line, 8–13 days from the 5th to 8th stages and 11–16 days for the remaining stages " D " line tumours exhibited an establishment time of 7–8 days up to the 3rd stage and thereafter varied from 11–16 days

The growth rates of the homologous tumours BP1 and MV212 and their respective irradiated lines are shown in Fig 3 and 4 No changes in tumour establishment times or growth rates were observed

DISCUSSION

The growth rate effects of serial irradiations on Sarcoma 37 were found to be the result of an intrinsic alteration in cell behaviour, as the observed changes were reproduced after sub-line passages without further irradiations These

findings are therefore comparable with those of Snellman (1935) on the Jensen rat sarcoma The increase in establishment time and decrease in growth rate were observed in both irradiated lines of Sarcoma 37, but no changes of this nature were observed in the two irradiated lines of the homologous tumours

A constant and permanent change in growth rate in all the irradiated tumours has therefore not been demonstrated in this study, however, it has been shown that a permanent change is possible This fact should therefore be taken into account where the assessment of radiosensitivity is based on tumour growth rate after a single irradiation dose, as employed by Dittrich, Hohne and Schubert (1956), particularly where non-strain-specific tumours are employed
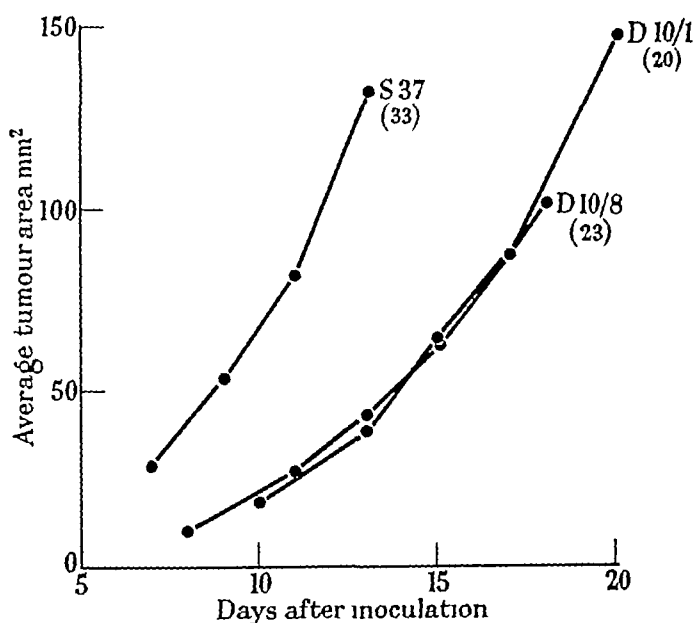
Fig 2 —Growth rates of Sarcoma 37 and its serially irradiated " D " line Figures in parentheses denote numbers of mice employed in each group

SUMMARY

1 Growth rate comparisons have been made on tumour transplants from untreated and serially irradiated lines of Sarcoma 37 and two homologous mouse tumours

2 Sarcoma 37 serially irradiated with sub-lethal doses showed an increase in tumour establishment time and a decrease in growth rate A similar but less marked effect was demonstrated when this tumour was serially irradiated with half-lethal doses at each stage

3 These growth changes were not altered by sub-line passages without further irradiation

4 Serially irradiated lines of two homologous tumours showed no variations in establishment time or growth rate when compared with their respective controls

My thanks are due to Mr F W Butcher and Miss G V Adam for assistance with the animal experiments
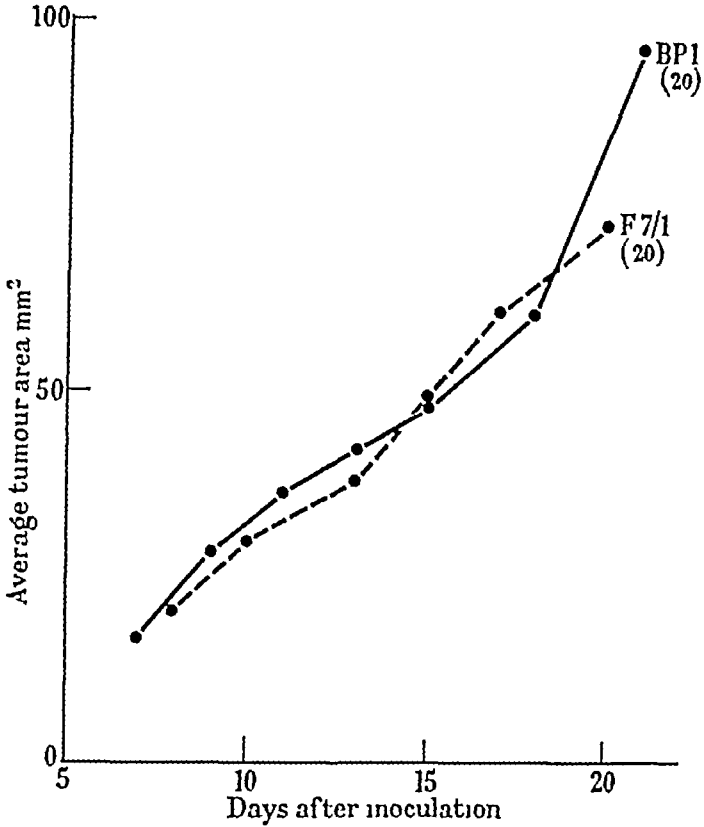
FIG 3 —Growth rates of spindle celled sarcoma BP1 and its serially irradiated " F " line
Figures in parentheses denote numbers of mice employed in each group
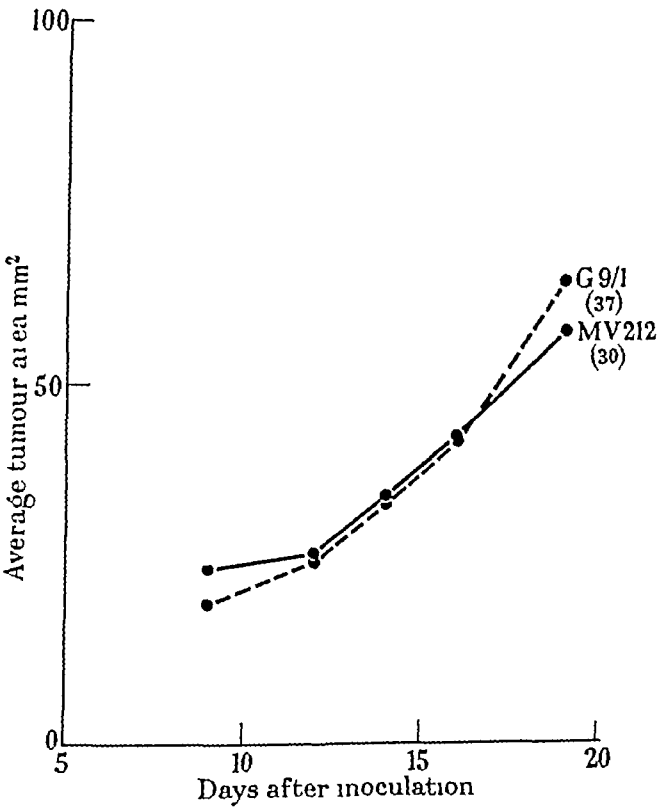
FIG 4 —Growth rates of mammary adenocarcinoma MV212 and its serially irradiated " G "
line Figures in parentheses denote numbers of mice employed in each group

The expenses of this research were defrayed from a block grant by the British Empire Cancer Campaign

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THE EFFECT OF SARCOMA 37 ON THE INTRACELLULAR DISTRIBUTION OF MOUSE LIVER CATALASE

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MUCH work has been published on the effect on liver catalase of tumour growth, and of the injection of whole or semi-purified tumour homogenates, but in all these studies the sole criterion of activity has been a simple depression in catalase level An increasingly large number of substances apparently unrelated to tumour tissue, have been shown to depress liver catalase activity on injection, e g methyl *bis* (B-chlorethyl) amine, 9 10-dimethyl-1 2-benzanthracene, butter yellow and its 3'methyl demative, 4'-amino-2 3-dimethylaminoazobenzene, B-naphthylamine, benzpyrene (Adams and Roe, 1953), homogenised normal spleen tissue (Day, Gabrielson and Lipkind, 1954), aminotriazole (Heim, Appleman and Pyrform, 1955) allyl isopropyl acetyl carbamide (Schmidt, Figen and Schwarz, 1955), cysteine and a number of its derivatives (Hirai and Deutsch, 1958), thiourea (Margoliash, 1958, personal communication), testosterone and 17-methyl testosterone (Adams, 1960)

There is little direct evidence about the mechanism by which these substances depress catalase activity *in vivo* Probably none of them directly inhibit the enzyme Amino triazole acts *in vitro* by combining with catalase-hydrogen peroxide Complex I (Margoliash and Norogrodsky, 1958) However this substance is characterised *in vivo* by an extremely rapid action—liver catalase activity being reduced almost to zero in a few hours It is well known that hydrogen peroxide destroys catalase and therefore one possible explanation is that these substances affect catalase activity through the production of hydrogen peroxide during their metabolism Certainly if every substance which gives rise to H_2O_2 *in vivo* depresses catalase activity, such depressions are completely non-specific Has then tumour growth or tumour tissue any specific action on liver catalase ?

Adams and Burgess (1959a) recently showed that when liver slices were incubated *in vitro* in a phosphate saline medium, catalase migrated from the large granules into the extra particulate cytoplasm (EPC) This observation led to the suggestion (Adams, 1960) that such catalase migration may occur *in vivo* and that catalase is synthesised only in the large granules On this view the EPC level would be maintained by catalase derived from the granules by migration through the granule membranes If this is correct, then the permeability of the granule membranes will be one of the factors determining the EPC catalase level Adams (1960) also suggested that, in the simplest analysis, the granule/EPC catalase distribution ratio would provide an approximate index of granule permeability He showed that after the injection of cysteine, methyl butter yellow, or thiourea, depressions in catalase activity were observed in both granule and EPC

fractions, but that these changes occurred without alteration in the granule/EPC distribution ratio   Further evidence in the same paper showed that androgenic hormones altered the granule/EPC distribution ratio in a way consistent with the supposition that they act by increasing the permeability of the large-granule membranes to catalase   Adams (1951) found that there appeared to be an antagonism between the action of testicular and adrenal hormones, and that of tumour homogenate or catalase activity

The purpose of this paper is to make a preliminary study of the action of tumour growth and tumour homogenate on intracellular catalase distribution

MATERIALS AND METHODS

Animals —Young adult mice of the 101 and CBA strains and of an albino strain derived from AKR were used   These animals were all bred in this laboratory by brother-sister mating, but some AKR-substrain mice were obtained direct from the Laboratory Animal Bureau

Preparation of liver fractions and estimation of liver catalase activity —Catalase estimations were made on whole liver homogenates, and on granule and EPC fractions   The method of estimation of catalase activity has been fully described in previous publications (Adams, 1950, 1952) and the preparation of the homogenates and fractions by Adams and Burgess (1957, 1959a)   Ethanol (final concentration 0 01 M) was added to all catalase containing solutions to prevent loss of catalase due to " Complex II " formation (Chance, 1950   Adams and Burgess 1959b)

Tumour —Sarcoma 37 was obtained originally from the Imperial Cancer Research Fund Laboratories and maintained by serial passage in this laboratory

Triton × 100 —This non-ionic detergent (kindly given to us by Charles Lennig & Co) was used at a final concentration of 0 25 per cent v/v to disrupt large granules and liberate their catalase activity into solution

RESULTS

The injection of normal tissue

Adams (1950), working with homogenates containing principally EPC fraction found that the injection of 50 mg doses of normal tissue did not depress catalase activity significantly   In view of the finding by Day *et al* (1954) that injections of homogenised spleen did depress liver catalase activity   the effect of larger doses of normal tissue on total granule, and EPC catalase was investigated   The normal tissue used was a mixture of the liver spleen and kidneys taken from sufficient CBA mice to provide the total required   The mixed tissues were homogenised (50 strokes with a Ten Broeck grinder) and injected subcutaneously in 100 and 200 mg doses into AKR-substrain female mice   As shown in Fig 1 the lower dose produced only a slight depression in catalase activity   but the higher dose resulted in considerable depression in catalase activity in both granule and EPC fractions   However, the granule EPC catalase distribution ratio remained almost unaltered through the course of the experiment   Fig 1 also shows that the effect of a single injection of homogenised rat liver (100 mg) was similar to that produced by the same dose of mouse tissue   Closely similar result i.e depression in catalase activity with little or no alteration in the distribution ratio

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were obtained after the injection of cysteine, thiourea, and butter yellow (Adams, 1960)

The injection of tumour tissue

Fig 2 shows the result of the injection of 100 mg and 200 mg of homogenate prepared in a similar way from Sarcoma 37 into AKR-substrain females As with normal tissue, depressions in catalase activity were observed, but with the
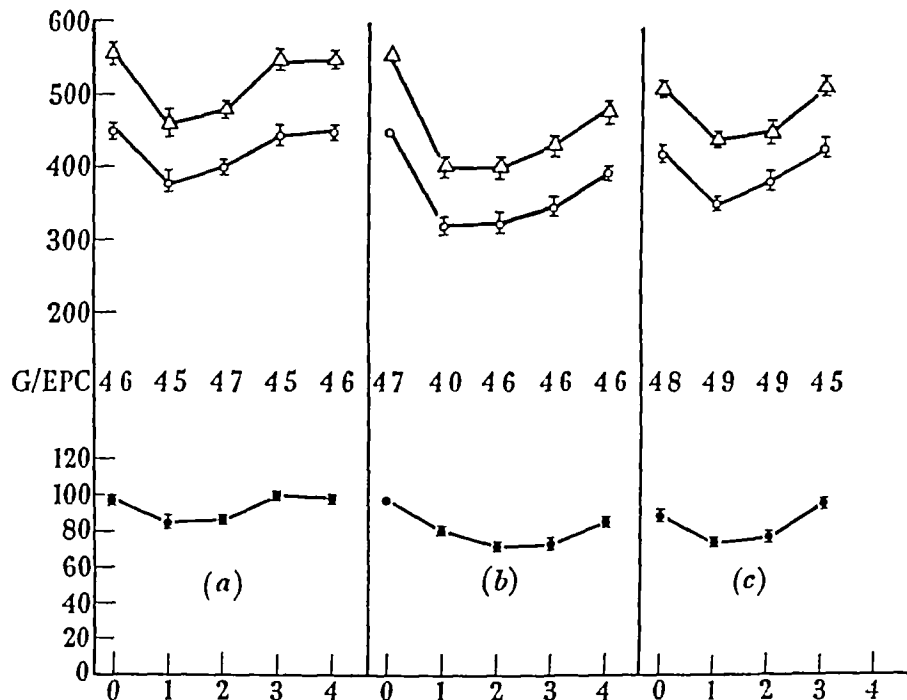
FIG 1 —The effect of the injection of homogenised normal tissue on the catalase activity and granule/EPC catalase distribution ratio of AKR substrain ♀ mice   (a) and (b) 100 mg and 200 mg of mixed liver kidney and spleen from CBA mice   (c) 100 mg of similar tissue from albino rats   Injection at 0 days

△————△ Total catalase   O————O Granule catalase
●————● EPC catalase

Results are expressed in this and subsequent figures as arithmetic means ± Std errors of means   Twelve control and eight treated animals/group
Ordinate —Catalase activity in arbitrary units/mg N
Abscissae —Time in days

difference that the granule/EPC catalase distribution ratio increased considerably Fig 3 shows the results of the injection of 100 mg of normal tissue, and 100 mg of tumour tissue, into CBA males   The normal tissue resulted in depression in catalase activity without alteration in the granule/EPC distribution ratio (3 3 in normal males)   The injection of tumour homogenate resulted in an increase in the ratio to 4 6

Tumour growth

The effect of tumour growth on the catalase activity and granule/EPC distribution ratio in AKR-substrain female and 101 male and female mice is shown
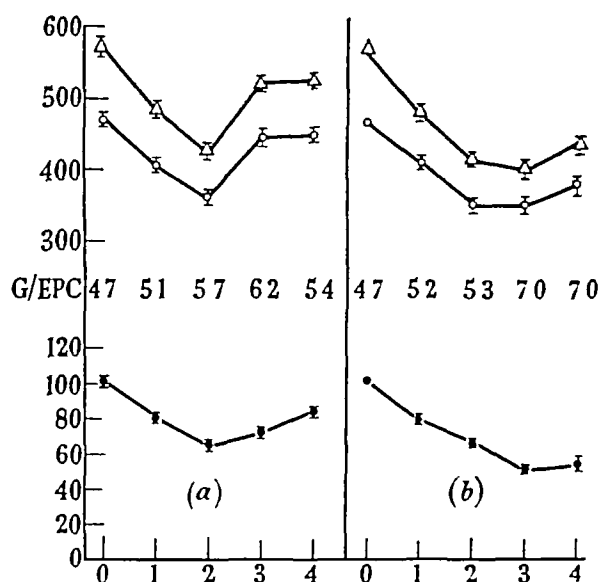
Fig 2 —The effect of the injection of homogenised S37 tissue on the catalase activity and granule/EPC catalase distribution ratio of AKR substrain 2 mice   (a) 100 mg   (b) 200 mg injected at 0 days

△————△ Total catalase   O————O Granule catalase
●————● EPC catalase

Ordinate —Catalase activity in arbitrary units/mg N
Abscissae —Time in days
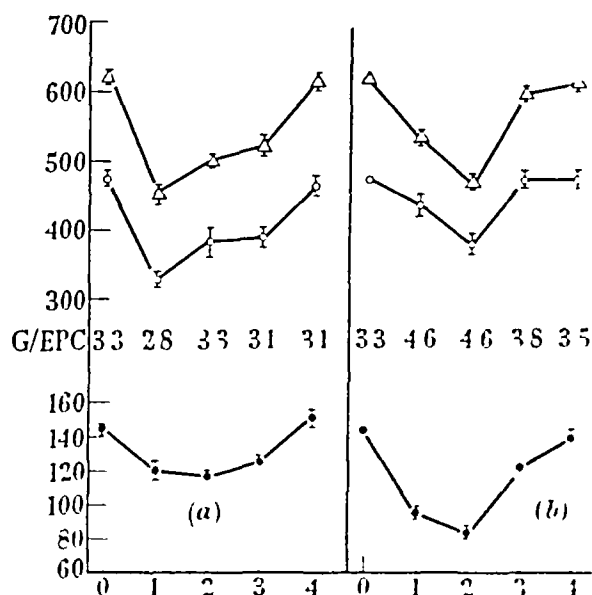
Fig 3 —The effect of the injection of homogenised tissue on the catalase activity and EPC catalase distribution ratio of CBA mice
    (a) 100 mg liver kidney spleen taken from AKR mice
    (b) 100 mg S37 tissue

————△ Total catalase   ———— Granule catalase
●————● EPC catalase

Ordinate —Catalase activity in arbitrary units/mg N
Abscissae —Time in days

in Fig 4 Progressive decreases in catalase activity in both granules and EPC were associated with progressive increases in the granule/EPC distribution ratio In the AKR-substrain mice considerable tumour necrosis was found, but little or none in the 101 mice

DISCUSSION

Surprisingly enough perhaps comparatively little work has been done on direct comparison of the effect of the injection of normal and tumour tissue on liver catalase Adams (1950) (measuring principally EPC catalase) found that
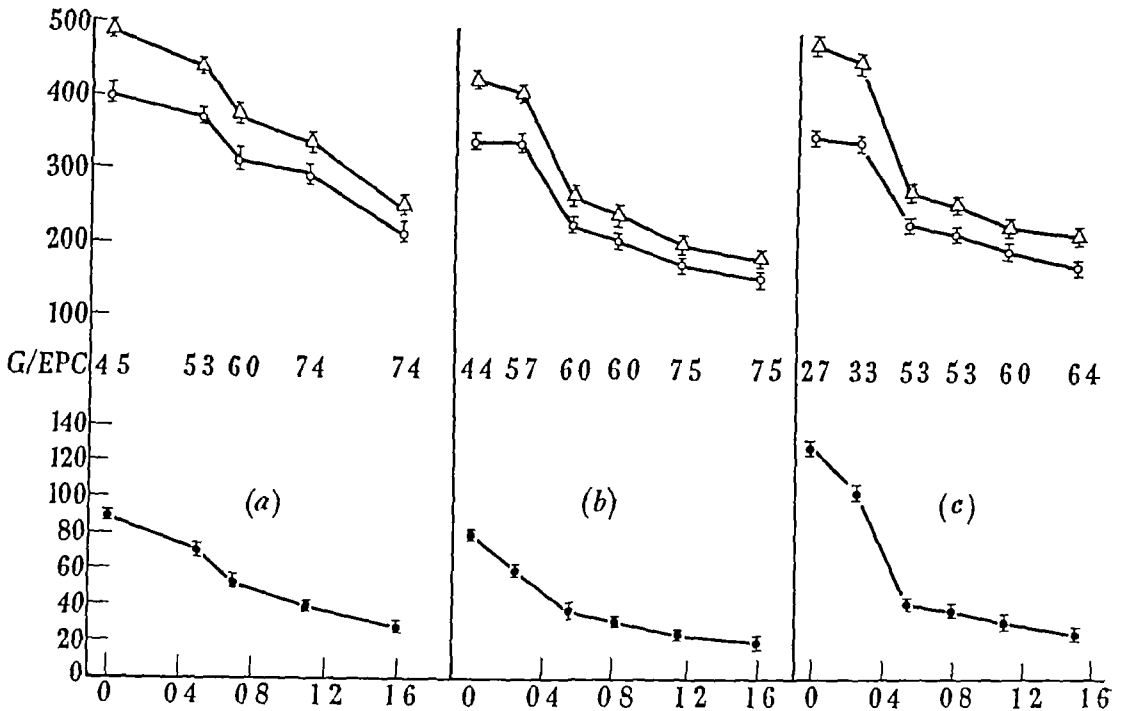
Fig 4 —Effect of S37 tumour growth on catalase activity and granule/EPC catalase distri
bution ratio of (a) AKR substrain ♀ mice   (b) 101 ♀ mice   (c) 101 ♂ mice   The tumour
bearing mice were formed into groups of 6-8 animals bearing tumours of similar size and the
tumour weights averaged to give the points on the graphs

△————△ Total catalase   ○————○ Granule catalase
●————● EPC catalase

Ordinate —Catalase activity in arbitrary units/mg N
Abscissae —Tumour weight in grams

the injection of 50 mg dose of normal tissue did not depress mouse liver catalase significantly, but 50 mg dose of S37 did   The present work shows clearly that catalase depressions may easily be obtained after the injection of normal tissue, provided that the dose is sufficiently high This depression of catalase activity by normal tissue injection confirms the result of Day *et al* (1954)   In fact if only *total* catalase activity is considered normal tissue homogenate may depress catalase to the same extent, dose for dose, as tumour homogenate The effect on the intracellular (granule/EPC) catalase distribution ratio was however quite different   Normal tissue homogenate did not alter this ratio in either sex, and this resembled thiourea, cysteine and methyl butter yellow in this respect (Adams,

1960) On the other hand, tumour homogenate increased the granule/EPC ratio considerably in both sexes, and the same phenomenon was seen during tumour growth The results suggest that injected tumour tissue has two separate actions on catalase Firstly, the total enzyme level is decreased, an effect which also occurs with normal tissue, and secondly, superimposed on this, there is an increase in the granule/EPC distribution ratio This action on the distribution ratio, which is apparently specific, shows itself principally by a decrease in the EPC level Thus if a comparison is made of the action of tumour homogenate and normal tissue homogenate on the EPC catalase level only, tumour tissue will produce a greater depression compared with normal tissue This accounts for the results previously reported (Adams, 1950) that 50 mg tumour homogenate significantly depressed the (EPC) catalase activity whereas no depression was caused by normal tissue homogenate

The results given here, taken in conjunction with those summarised in the introduction, make it clear that a simple catalase depression is not in the least a specific action of tumour tissue or of tumour growth

It seems also quite clear that the fractions derived from tumour tissue by various investigators cannot, on the available evidence, be said to contain any specific tumour agent These fractions have been tested solely on the basis of their ability to depress catalase activity *in vivo* after injection There are obviously many substances which could be present in varying amounts in tumour tissue and in normal tissue, which will produce a catalase depression when injected in adequate dosage

Tumour growth resulted in a progressive fall in catalase activity, and a progressive increase in granule/EPC distribution ratio However the catalase activities in these mice fell to lower levels than those observed after injections of tissue homogenates This point will be dealt with in a future publication

Adams (1960) showed that androgenic hormones injected into female mice exerted a specific effect in decreasing the liver catalase granule/EPC distribution ratio from the normal value of about 5 0, to the normal male value of about 3–3 This was interpreted as showing that the hormones increase the permeability of the granule membranes to catalase On the same view, the increase in distribution ratio caused by tumour growth and tumour tissue would result from a decrease in the permeability of the large-granule membranes to catalase The antagonistic action of tumour tissue and hormones reported by Adams (1951) appears therefore to result from their opposite effects on granule membrane permeability

SUMMARY

(1) Depression of liver catalase activity results equally well from injections of S37 homogenate or normal tissue homogenates

(2) S37 homogenate increases the granule/EPC catalase distribution ratio, but normal tissue does not

(3) It is concluded that S37 homogenate contains an agent which decreases the permeability of the granule membranes to catalase

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THE EFFECTS OF SOME POLYCYCLIC HYDROCARBONS ON MOUSE LIVER –SH LEVELS

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THE involvement of sulphydryl groups in the biological activity of carcinogenic agents is a subject which has frequently been discussed   Unfortunately, very few attempts have been made to measure any actual changes in tissue –SH levels induced by carcinogens

Estimations of the glutathione level of mouse skin after treatment with various hydrocarbons were made by Crabtree (1946)   The non-carcinogens, naphthalene, anthracene and phenanthrene caused distinct falls as compared with normal levels but dibenzanthracene and 3 4 benzpyrene had no effect   More recently DiPaolo and Niedbala (1957) could find no changes induced by anthracene in the –SH levels of dermis and epidermis of mouse skin   On the other hand they found 1 2 5 6 dibenzanthracene and 9 10 dimethylbenzanthracene to cause an initial rise, then a sharp fall followed by a further rise in level   This last rise persisted for as long as five days after treatment with the hydrocarbon

Estimations of water extractable –SH from normal and benzpyrene treated rat livers were made by Rondoni and Boretti (1947)   It was concluded that, at intervals between 24 hours and 21 days after treatment there was a fall in liver –SH in the treated animals   Later, however, Rondoni (1955) himself suggested that as the measured variations were very small the conclusions should be treated with reserve

Based on the argument that serum or plasma –SH levels should be a reflection of tissue levels Wood and Kraynak (1953) determined the plasma –SH level of dogs at intervals after intravenous injections of anthracene or 3 4 benzpyrene and the serum –SH levels of rabbits after similar treatments   Between ½ hour and 6 hours after treatment benzpyrene caused significant falls in –SH content but anthracene had no effect on the normal level

The present work records the results obtained from measurements of the liver –SH levels of mice at relatively short intervals after the intravenous injection of polycyclic hydrocarbons   Liver was selected for these studies as being an organ where hydrocarbons are known to be deposited and metabolised   It was thought that any variations found might help to throw light on the problems associated with questions of the metabolism of hydrocarbons

EXPERIMENTAL

All mice used in these experiments have been taken from a stock of inbred Strong A animals maintained in this laboratory   Each batch of mice used has

comprised animals born within 1–2 days of one another   The actual ages of the batches are recorded with the results

The hydrocarbons used were anthracene, pyrene, perylene, 1 2 5 6 dibenzanthracene and 3 4 benzpyrene   The hydrocarbons were prepared as colloids in distilled water   Each experimental animal received a single intravenous injection via the tail vein of 0 5 mg of hydrocarbon in 0 5 ml of distilled water

Liver –SH levels were determined by the method already described by Calcutt and Doxey (1959)   This involves the immersion of a known weight of liver slices (weights of 90–160 mg were used) in a measured volume of a standard solution of p-chloromercuribenzoic acid (C M B) and after an appropriate time interval for the reaction to take place the potentiometric titration of any unchanged C M B with a standard solution of cysteine hydrochloride   During the present work every titration has been run in duplicate, the two runs being done at the same time on completely separate sets of instruments   With certain exceptions during a run with dibenzanthracene (this will be referred to later) end points on the two sets of equipment were either completely in agreement or within 0 1 ml of one another   For calculation purposes the mean of the two results was used

RESULTS

The results are given under the headings of the individual hydrocarbons   For each batch of mice a mean figure and standard deviation for untreated animals has been determined   In each case 10 animals have been used for this purpose

Anthracene —Female mice aged 25 weeks   Control value 30 4 ± 2 8 μg of –SH per 100 mg wet weight of liver   Results are shown in Fig 1   Apart from a very sharp rise in –SH level occurring ½–1 hour after injection of the hydrocarbon, levels tend to fall consistently below the control value , this still being apparent 24 hours after treatment

Pyrene —Male mice aged 26 weeks   Control value 32 9 ± 3 5 μg of –SH per 100 mg wet weight of liver   Results are shown in Fig 2   After an initial rise in level similar to that found with anthracene the level falls below the control value for a few hours and then returns to the normal range

Perylene —Female mice aged 25 weeks   Control value 29 7 ± 4 6 μg of –SH per 100 mg wet weight of liver   Results are shown in Fig 3   Again there was an early rise (½ hour) in –SH level, but this time apparently succeeded by a further rise at about 2 hours after treatment   This was followed by a return to the normal or a possibly slightly enhanced level   At no time up to 24 hours after injection was there any decline in –SH level below the range of normal values

3 4 *Benzpyrene* —Female mice of the same batch as the previous experiment   The results are shown in Fig 4   As in the earlier experiments there was an initial rise in –SH value, but this time occurring at 1½–2 hours after treatment   This was followed by a fall to levels below the normal range and then a return to normal after about 12 hours

1 2 5 6 *Dibenzanthracene* —Male mice aged 10 weeks   Control value 32 1 ± 3 6 μg of –SH per 100 mg wet weight of liver   The results are shown in Fig 5   Initially the picture closely resembled that obtained with perylene , there being an early rise succeeded by another rise at about 3½–4 hours   This was succeeded by a fall to levels below normal and then a gradual return to a normal level by 24 hours   At 48, 72 and 96 hours after treatment, however, the
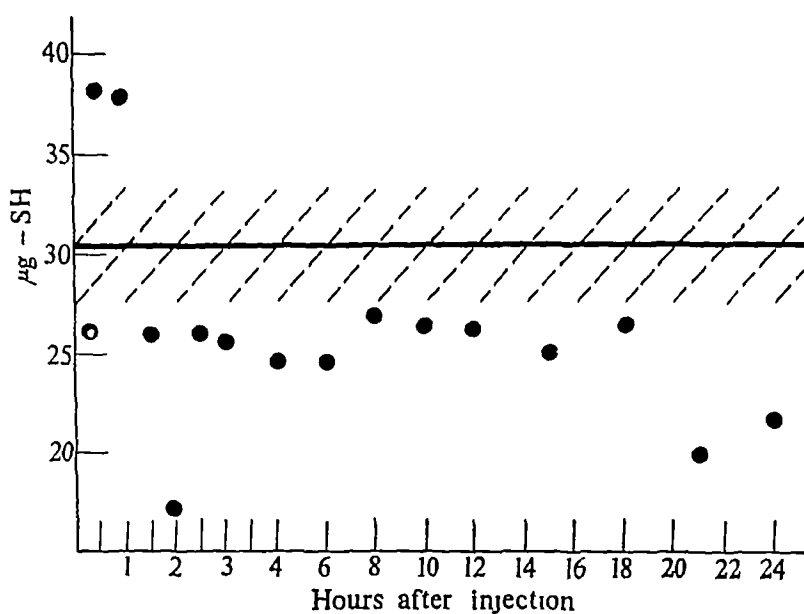
Fig 1 —Mouse liver -SH levels after treatment with anthracene

In this and all successive figures the mean control figure for untreated animals is shown by a heavy horizontal line , the standard deviation of the control figure is indicated by hatching and experimental points are shown as circles
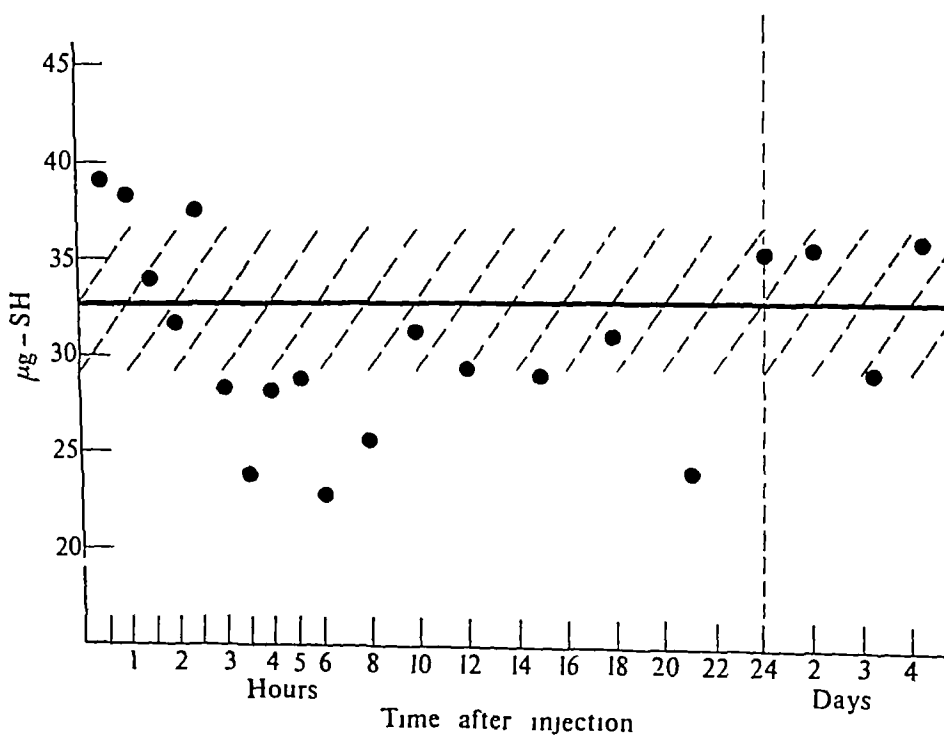

Fig 2 —Mouse liver -SH levels after treatment with pyrene

titration curves were unusual in that two sharp falls were recorded instead of the normal one associated with the end point   Calculated from the end points determined from the first falls in the curves enhanced -SH levels were found as
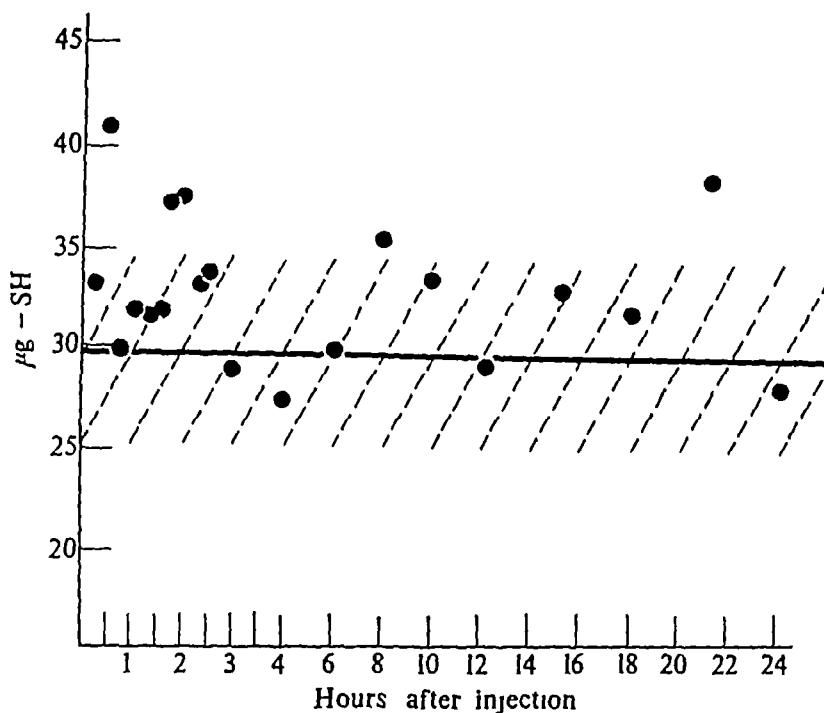
FIG 3 —Mouse liver -SH levels after treatment with perylene
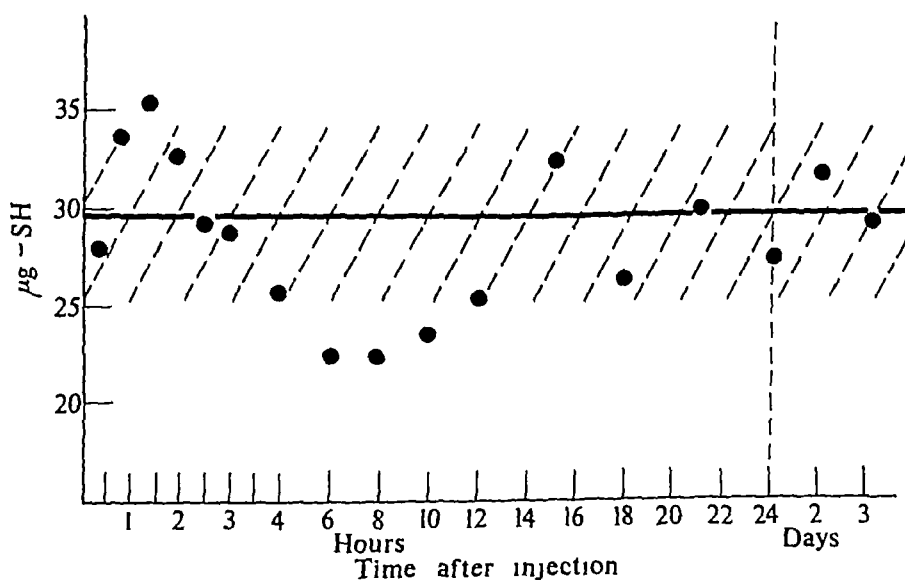
FIG 4 —Mouse liver -SH levels after treatment with 3 4 benzpyrene

shown in the figure   Calculations based upon the second end point, however, gave levels which fell strictly within the normal range

The other finding from this data is the close correspondence between the normal control levels in the different batches of mice   The male animals show a slightly higher level than the females

DISCUSSION

The hydrocarbons used in the present experiments fall into one or other of two groups   Anthracene, pyrene and perylene are apparently biologically inactive   Benzpyrene and dibenzanthracene, apart from being potent carcinogens, are also very active photosensitizing agents, effective in eliciting neural inductions and capable of causing mitotic abnormalities   Apart from Ilfeld's (1936) claim to have induced a hepatoma by the insertion of a pellet of dibenzanthracene into mouse liver there is no evidence suggesting any effect of any of the compounds used on liver tissue   It is against this background of the known
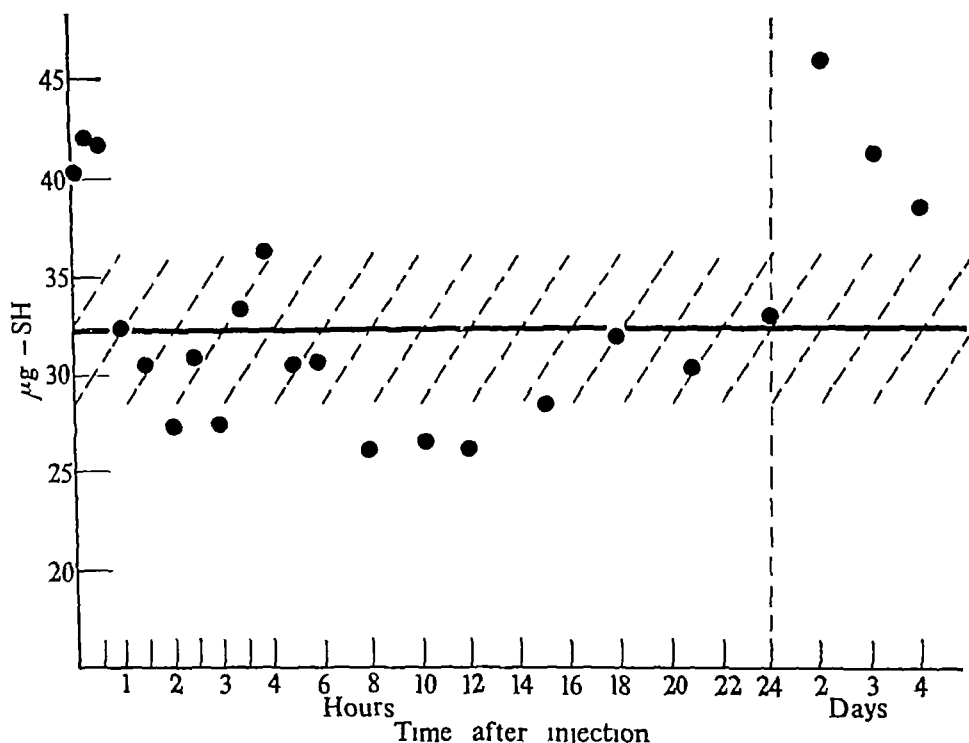
Fig 5 —Mouse liver –SH levels after treatment with 1 2 5 6 dibenzanthracene

biological activity and the known data in respect of metabolism that the present results must be considered

The one feature displayed by all the agents tested is the early induction of a rise in liver –SH level   This parallels the finding of DiPaolo and Niedbala (1957) that dibenzanthracene and 9 10 dimethylbenzanthracene cause an early rise in skin –SH levels   Since these last authors applied their hydrocarbon as solutions in acetone whilst we have used aqueous colloids this rise would not appear to be due to the vehicle of introduction but to an effect of the hydrocarbon   Further, the appearance of this rise within 15 minutes in the case of dibenzanthracene but only after 1–1½ hours in the case of benzpyrene is also consistent with this effect being due to the hydrocarbon, since the introduction of the agent and handling of the animals has been identical in the two series

After the initial rise the picture varies among the different compounds used   Both pyrene and benzpyrene show a fall to levels below the normal range   Dibenzanthracene shows a fall, then a rise and a further fall   Perylene drops to

a level which may be slightly above the normal whilst anthracene settles to a level rather below the normal figure   There is, obviously, no relationship between these behaviours and the carcinogenic or other biologic activity of the compounds concerned

With regard to metabolism, both pyrene and benzpyrene are known to be converted to phenolic derivatives in mouse liver (Harper, 1958a, 1958b)   In the case of dibenzanthracene Dobriner, Rhoads and Lavin (1939) isolated a phenolic derivative from mice and this was shown by Cason and Fieser (1940) to be 4′–8′ dihydroxydibenzanthracene   Breakdown products of this derivative were also found by Heidelberger and Wiest (1951)   In the case of perylene nothing is known with regard to metabolism   Anthracene metabolism has been extensively investigated in rats and rabbits but not in mice   However, Calcutt (1959, unpublished data) isolated a derivative, apparently identical with the 1 2-dihydroxy-1 2-dihydro anthracene formed by rats, from both the urine and livers of mice treated with anthracene   It is known that anthracene in rats is partially excreted as a mercapturate (Boyland and Levi, 1936)   If a similar mercapturate formation occurs in mice then this may account for the persistent low level obtained for –SH values after treatment with this hydrocarbon

At the moment no positive conclusions can be drawn as to any relationship between fluctuations in liver –SH levels and metabolism   Further data is required in regard to the intracellular sites of –SH changes and sites of metabolic activity

The anomalous findings in respect of dibenzanthracene at 48, 72 and 96 hours after treatment are, at the moment, unexplained

The results obtained in the present series are not strictly comparable with previously published work since different tissues and time intervals have been used   It may, however, be noted that the picture of an initial rise followed by a fall and then a further rise is very similar to that achieved in skin with dibenzanthracene and dimethylbenzanthracene by DiPaolo and Niedbala (1957)   On the basis of the presently available evidence there appears to be no correlation between carcinogenicity of the applied agent and resulting disturbances of sulphydryl level, but there may be an association with metabolic behaviour

SUMMARY

The sulphydryl levels in the livers of mice which had received intravenous injections of 0 5 mg of a polycyclic hydrocarbon have been determined Anthracene, pyrene, perylene, 3 4 benzpyrene and 1 2 5 6 dibenzanthracene were used

All five hydrocarbons caused an initial rise in liver –SH level   This occurred at various times between 15 minutes and $1\frac{1}{2}$ hours after treatment   Subsequent to this rise there were fluctuations in –SH level , these varying with the hydrocarbon used

The results are discussed in relation to the known biologic activities and metabolism of the hydrocarbons

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THE INTERMEDIARY METABOLISM OF POLYCYCLIC HYDROCARBONS

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PREVIOUS studies of the metabolism of pyrene and 3 4-benzpyrene have been reported (Harper, 1957a, 1958a, 1958b, 1958c) This work confirmed the earlier finding of Weigert and Mottram (1946) that, following injection of 3 4-benzpyrene into mice, two metabolic fractions, designated as X_1 and X_2, are excreted in the bile and undergo conversion to phenolic derivatives during passage through the intestine These two fractions were identified respectively as sulphuric acid (X_1) and glucuronic acid (X_2) esters of fully aromatic benzpyrenols (Harper, 1958b, 1958c) and a similar sequence of conjugation and hydrolysis was established for the 3-pyrenol metabolite of pyrene (Harper, 1958a, 1958c) Also associated with the glucuronide fractions yielded by both pyrene and 3 4-benzpyrene was an acid-decomposable precursor of the parent hydrocarbon

This work has now been extended to a range of hydrocarbons, namely 1 2-benzanthracene, chrysene, 20-methylcholanthrene, 1 2 5 6-dibenzanthracene and anthracene, utilising the same extraction and analytical procedures Full details of these are to be found in the above publications

MATERIALS AND METHODS

20-Methylcholanthrene (Hoffmann-La-Roche and Co Ltd) and anthracene (B D H Ltd) were used as purchased , the other hydrocarbons were purified by chromatography on alumina from benzene and cyclohexane followed by fractional crystallisation from ethanol, aq ethanol or benzene Colloidal solutions of the hydrocarbons were prepared by the method of Boyland (1932) In the case of 1 2 5 6-dibenzanthracene and chrysene however it was found necessary to heat both acetone solution and water to 50° C prior to mixing

Strong A mice, in batches of about twenty, were injected intravenously with 0 5 mg of colloidal hydrocarbon and the distribution of metabolites within the internal organs of the body was then investigated using the same general methods of extraction and chromatographic separation previously reported for pyrene and 3 4 benzpyrene A modification adopted in the case of faecal metabolites however was as follows

The freshly voided faeces were extracted into xylene via acetone (Harper, 1957a) and the xylene passed through a column of silica gel (100/200 mesh) for the removal of acidic metabolites and much of the colouring matter The filtrate from the column was evaporated under reduced pressure, the residue extracted repeatedly with boiling cyclohexane and the cyclohexane chromatographed on

silica gel   Any phenolic metabolite of the hydrocarbon was then retained as a blue fluorescent zone on the column from which it was readily eluted either with ethanol or with cyclohexane/benzene mixtures   This procedure was preferred to chromatography on alumina as used in previous studies owing to the difficulty experienced in removing small amounts of phenolic derivatives from this adsorbent

4'-Hydroxy-1 2-benzanthracene (4'-benzanthrol) was synthesised by the method of Sempronj (1939)   4'-Methoxy-1 2-benzanthracene was prepared from this by methylation using dimethyl sulphate and excess sodium hydroxide, the reaction being continued until the bright golden yellow fluorescence of the solution had disappeared

1-Anthrol was prepared by reduction of 9 10-anthraquinone-1-sulphonic acid followed by alkali fusion

The reference conjugates, 4'-benzanthryl glucuronide and sulphate and 1-anthryl glucuronide and sulphate, were isolated from the bile and small intestines of mice injected with the parent phenols (cf Harper, 1958c)

RESULTS

Three hydrocarbons, 1 2-benzanthracene, chrysene and 20-methylcholanthrene, yielded well defined blue fluorescent X_1 and X_2 type metabolites   Excretion was mainly via the bile and hydrolysis to phenolic derivatives and, in the case of 20-methylcholanthrene, also to an acidic derivative, occurred in the caecum and large intestine   Blue fluorescent X_1 and X_2 type metabolites were also isolated from the bile and small intestine after injection with dibenzanthracene but the yields of these were greatly reduced by comparison with the other hydrocarbons   In marked contrast to this behaviour, however, was the failure of anthracene to yield any material that was recognisable as a metabolite under the same conditions of extraction and analysis

The X_1 type metabolites were readily hydrolysed by cold dilute mineral acid and by takadiastase β-Glucuronidase was without effect   The X_2 type metabolites on the other hand, were unaffected by treatment with mineral acid in the cold but hydrolysis occurred on prolonged heating under nitrogen with strong acid (6N HCl)   Rapid hydrolysis also occurred on incubation with β-glucuronidase but this effect was nullified in the presence of 10^{-2} M boiled saccharate solution

In view of these properties, and by analogy with the behaviour of the conjugates of 3-pyrenol and benzpyrenols (Harper, 1958c), the two metabolic fractions were concluded to be sulphuric and glucuronic acid esters respectively The essential problem therefore was the identification of the hydrocarbon moieties of the conjugates

(A) 1 2-Benzanthracene

The identification of the phenolic derivative of 1 2-benzanthracene excreted in the faeces of rats and mice was achieved by methylation and spectroscopic comparison with known methoxy-benzanthracenes (Berenblum and Schoental, 1943)   On this basis the 4'-methoxy derivative was selected as being identical with the methylated metabolite although the presence of absorbing impurity limited comparison to the long wave systems (above 300 mμ) of the absorption spectra   A characteristic feature of the spectrum was a band at 391 5 mμ in

hexane but, perhaps surprisingly, the graphical data reported for the methylated metabolite does not include the sharp prominent maximum at 311 5 mμ (Fig 2)

In the present work attempts have been made to confirm this identification by comparison of the conjugated and free phenolic metabolite with the synthetic analogues derived from 4'-benzanthrol

(a) *The conjugated metabolites*

The physical and chemical properties of the X_1 and X_2 type metabolites were respectively identical with those of 4'-benzanthryl sulphate and glucuronide
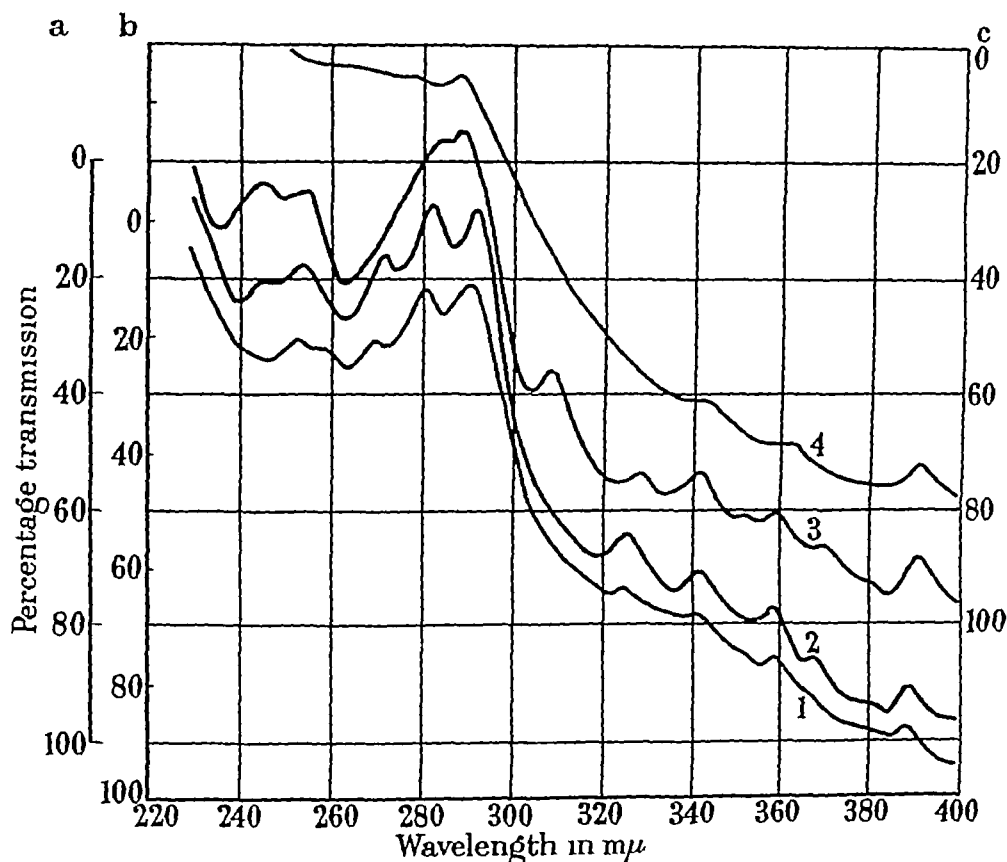
FIG 1 —Absorption spectra in ethanol

1 X_1 type metabolite of benzanthracene (b ordinates)
2 4' benzanthryl sulphate (a ordinates)
3 4' benzanthryl glucuronide (c ordinates)
4 X_2 type metabolite of benzanthracene (c ordinates)

The one exception was observed with the X_2 type metabolite for, on acid hydrolysis, a small amount of free 1 2-benzanthracene was liberated in addition to a phenolic derivative   The presence of an acid-decomposable precursor of the hydrocarbon was therefore indicated

The absorption spectra of the two sets of conjugates are recorded in Fig 1 The resemblance between the two types of spectra is at once apparent and provides confirmatory evidence of the conjugated nature of the hydrocarbon metabolites   In Table I however are recorded the detailed positions of the absorption bands and reference to this data reveals that slight differences exist

in the location of certain bands   (Failure to record a band at or near 308 mμ in the spectrum of the X_2 type metabolite is possibly due to the presence of absorbing impurity   This was in fact present in one sample of the metabolite isolated from stored bile)

A possible explanation of these differences is that the hydrocarbon metabolites consist predominantly of the conjugates of 4'-benzanthrol but that smaller amounts of other conjugated derivatives are also present   In the case of the glucuronide fraction this could be the hydrocarbon precursor but, as the sulphate yields only a phenolic fraction on hydrolysis, the presence of an additional benzanthrol is indicated

(b) The phenolic metabolite

The phenolic derivative of 1 2-benzanthracene isolated from the faeces could not be obtained free from absorbing impurity and this prevented complete characterisation by absorption spectroscopy   In the long wave region above 320 mμ however, bands characteristic of 4'-benzanthrol were present and consistent with this behaviour was the bright golden yellow fluorescence exhibited by the metabolite in sodium hydroxide   In view of the possibility of an additional benzanthrol being present the metabolite was subjected to methylation and chromatographic fractionation in the manner described for methoxybenzpyrenes (Harper, 1958b)   No marked differences were observed in the spectra of the fractions thus obtained and, on pooling and concentrating in vacuo, the absorption spectrum shown in Fig 2 was recorded   Also shown in Fig 2 is the spectrum of the phenolic derivative liberated from the sulphate conjugate by mild acid hydrolysis and the two are compared respectively with those of 4'methoxy- and 4'-hydroxy-1 2-benzanthracene   The detailed positions of the absorption bands are given in Table I

TABLE I

Absorption bands in ethanol (m μ)				Absorption bands in hexane (m μ)			
BAX_1	4'-BA-S	BAX_2	4'-BA-G	BA-OH	4'-OH	BA-OMe	4'-OMe
—	—	—	—	226	230	228	231
[246-248]	[244-248]	—	244-246	—	245	246	245-246
252	254	—	254	253	253 5	250-254	253 5
—	—	—	—	[262]	—	—	—
270	272	—	—	[270-272]	—	—	—
280	282	—	[284-286]	[278-280]	277	[280-282]	277
290-291	292	288	288	286	286	287-288	287 5
—	—	—	—	297	297	—	299
—	—	—	308	—	309 5	—	311 5
324	326	—	328	[320-324]	326-327	[326-328]	327
—	—	—	—	337	—	—	—
342	342	[338-342]	342	[340-341]	342	342	342 5
—	—	—	354-356	354-355	353	354	352-353
358	359	[358-362]	358-360	—	359	[358-360]	359-360
—	366-368	—	370	372	371-372	370-372	371
—	—	—	—	—	382	—	381
388	389	392	391-392	394	392	392	391 5

BAX_1 and BAX_2 = X_1 and X_2 metabolites of 1 2-benzanthracene
4'-BA-S and 4'-BA-G = sulphuric (S) and glucuronic (G) esters of 4'-benzanthrol
BA-OH = free phenolic component of the sulphate conjugate (BAX₁)
BA-OMe = methylated faecal metabolite
4'-OH and 4'-OMe = free (- OH) and methylated (-OMe) 4'-benzanthrol

Reference to this data reveals that, above 320 mμ, the spectra of the methylated metabolite and 4'-methoxy-1 2-benzanthracene are very similar and is in agreement with the conclusion of Berenblum and Schoental (1943) that hydroxylation of the benzanthracene nucleus occurs in the 4'-position It will be recalled that this wave length region only was utilised by these workers for their analysis In the present work however, the full absorption spectra have been recorded and below 320 mμ it is at once apparent that marked differences exist The major band of 4'methoxy-1 2-benzanthracene at 287 mμ is present
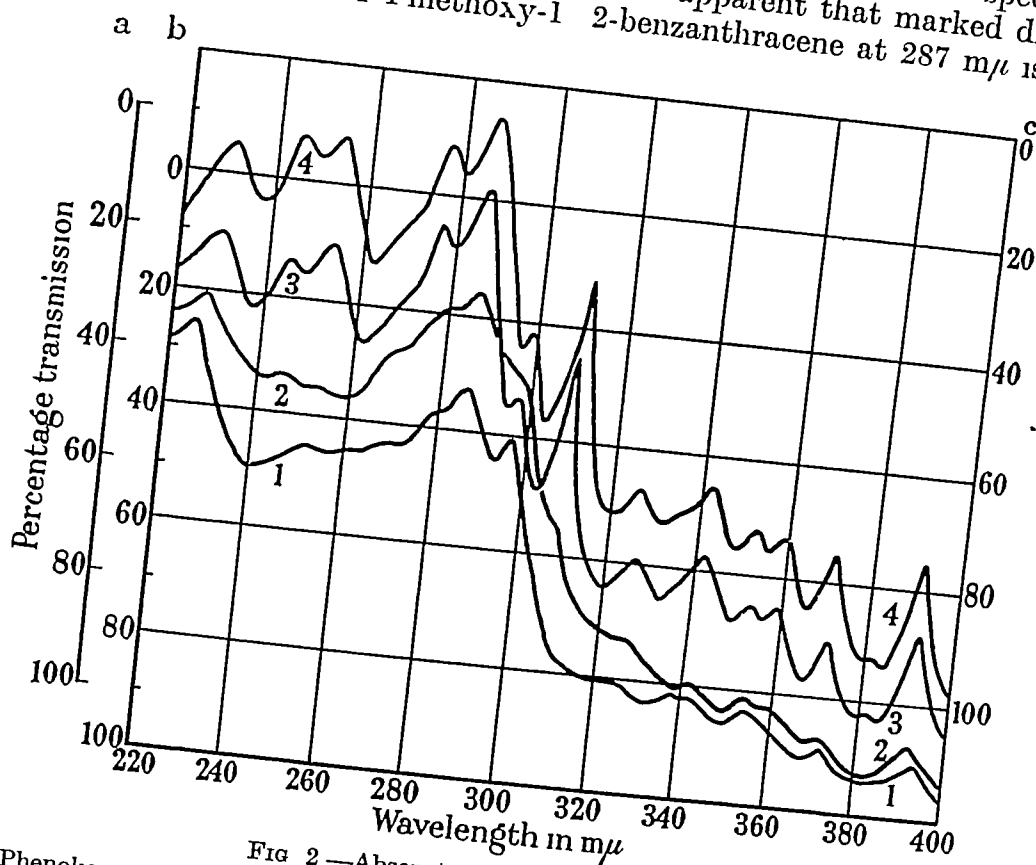
FIG 2 —Absorption spectra in hexane

1 Phenolic component of the X₁ type metabolite of benzanthracene (b ordinates)
2 Methylated faecal metabolite (b ordinates)
3 4' benzanthrol (a ordinates)
4 4' methoxybenzanthracene (c ordinates)

in the "metabolic" spectrum but a conspicuous feature is the absence of any band, other than a slight inflection, at 311 5 mμ The latter is a prominent feature of the 4'-substituted derivatives and is readily detected in all metabolic studies with these compounds It is doubtful therefore that failure to record this band is due to the presence of masking impurity of natural origin A possible explanation is that the presence of an additional related methoxylated benzanthracene interferes with the absorption of the 4'-methoxy derivative in this region although chromatographic fractionation revealed no evidence of this Support for this proposal however is provided by the spectrum of the free phenol liberated from the sulphate conjugate This is similar to that of 4'-benzanthrol in its general appearance but differences exist in both short and long wave regions An unknown phenolic derivative with bands at 226, 286, 297, 320–324,

337, 354–355, 372, and 394 mμ would indeed appear to represent the major component of the mixture

In conclusion, therefore, it can be said that the evidence obtained in this work is consistent with the view that a mixture of phenolic derivatives is formed as a result of the biological hydroxylation of 1 2-benzanthracene in the mouse One of them is 4'-benzanthrol, the other is neither 3-benzanthrol (Jones, 1945) nor 9 10-dihydroxy-1 2 -benzanthracene (Berenblum and Schoental, 1943) and is as yet unidentified By analogy with other hydrocarbons however it may logically be expected to be the 2'-hydroxy derivative
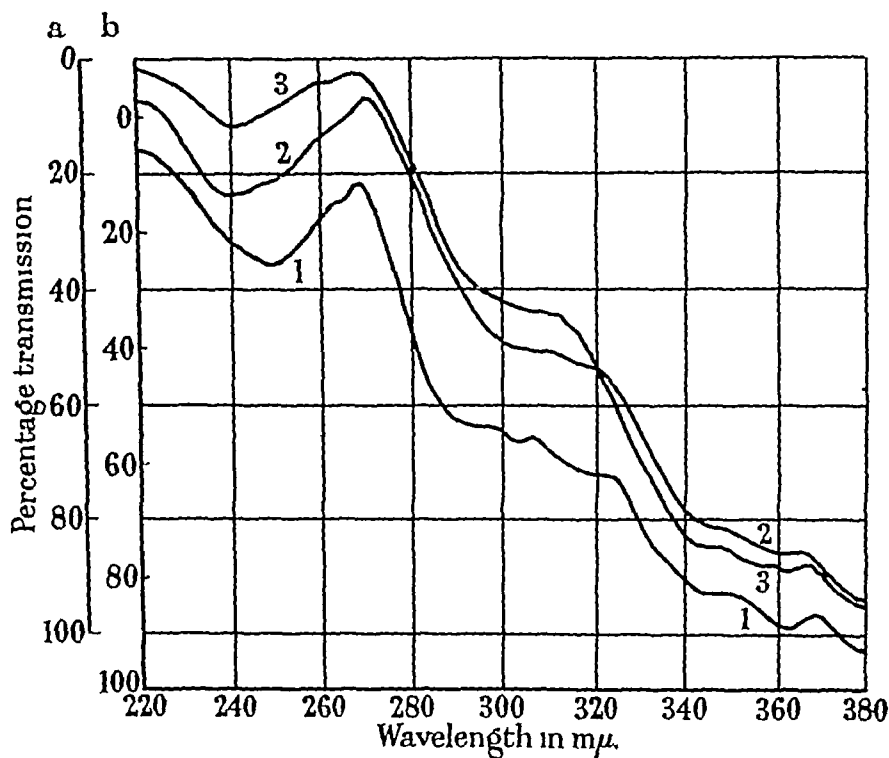
Fig 3 —Absorption spectra in ethanol

1 Phenolic metabolite of chrysene (b ordinates)
2 X$_1$ type metabolite of chrysene (a ordinates)
3 X$_3$ type metabolite of chrysene (a ordinates)

(B) Chrysene

The phenolic derivative excreted in the faeces after intraperitoneal injection of chrysene in the rat was identified by Berenblum and Schoental (1949) as 3-chrysenol Attempts to confirm this in the present work with mice have been only partially successful owing to the low yield obtained and difficulty experienced in purification The experiments have shown however that the two conjugated fractions yield what appears to be the same chrysenol on hydrolysis with takadiastase and β-glucuronidase respectively and that a small amount of free chrysene is also liberated from the glucuronide fraction on hot acid hydrolysis

The absorption spectra of the metabolites (Fig 3), although ill-defined, are consistent with a fully aromatic chrysenoid configuration The conjugated metabolites are therefore concluded to be (3)-chrysenyl sulphate and glucuronide respectively

(C) 20-Methylcholanthrene

This hydrocarbon (I) was of particular interest in its divergence from the fully aromatic configurations so far studied   Although existing evidence on the
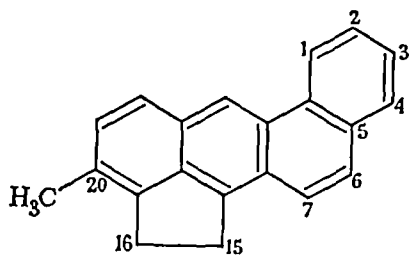
I

metabolism of this compound suggested that hydroxylation occurs on the aromatic benzanthracene nucleus (Cason and Fieser, 1940 , Dobriner, Rhoads and Lavin, 1942), general metabolic considerations were in favour of both phenol
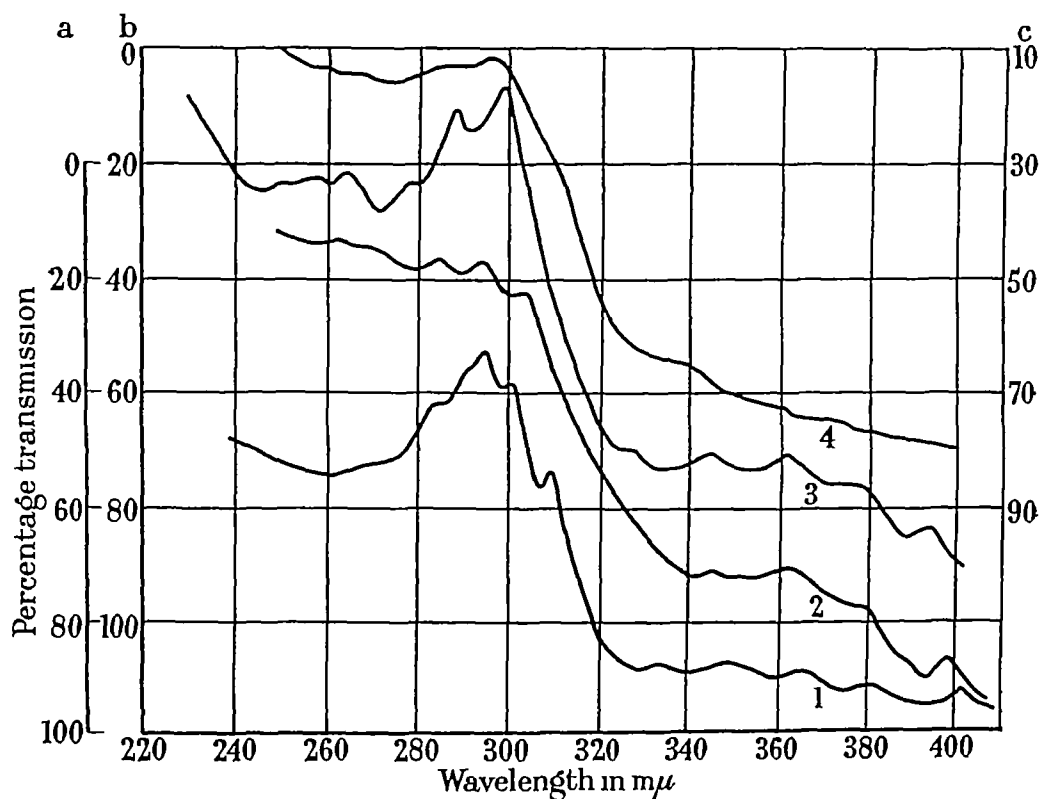
Fig 4 —Absorption spectra in ethanol

1 Carboxylic acid metabolite of 20 methylcholanthrene (a ordinates)
2 Phenolic metabolite of 20 methylcholanthrene (a ordinates)
3 X_1 type metabolite of 20 methylcholanthrene (b ordinates)
4 X_2 type metabolite of 20 methylcholanthrene (c ordinates)

and carboxylic acid formation   The latter may be expected to arise from the oxidation of the attached methyl group—cf the metabolism of 2-methylnaphthalene (Grimes and Young, 1956)—or from fission of the 15–16 bond—cf the

metabolism of acenaphthene (Chang and Young, 1943) This theoretical prediction has been borne out experimentally for two methylcholanthrene derivatives, one phenolic and the other acidic, have been isolated in this work These were absent from the bile and small intestine but were present in the caecum, large intestine and faeces

The phenolic derivative possessed normal chromatographic behaviour and the typical fluorescence colour change from blue to yellow occurred on the addition of sodium hydroxide to its ethanolic solution   The absorption spectrum shown in Fig 4 possessed bands at 284, 294, 304–305, 344–346, [352], 363 [378–380] and 398 mμ It has not been possible to identify it but, by analogy with other hydrocarbons, it may logically be expected to be either the 2- or 4-hydroxy derivative

The acidic derivative was not adsorbed on silica gel from xylene or benzene but was tightly held as a narrow blue fluorescent zone at the surface of an alumina column Elution was achieved with ethanol containing 1 per cent hydrochloric acid, ethanol by itself being ineffective A solution of the metabolite in this mixture possessed a yellow fluorescence which changed to bright blue on making alkaline with sodium hydroxide Its acidic nature was further emphasised by its solubility in dilute sodium bicarbonate In view of these properties the metabolite was concluded to be a carboxylic acid derivative As the absorption spectrum shown in Fig 4, with bands at 284, 295, 302, 312, 334, 348–350, 364–366, 378–380 and 402 mμ, establishes the integrity of the aromatic benzanthracene nucleus, three possible structures for the metabolite are

(a) Cholanthrene-20-carboxylic acid (II), arising from oxidation of the methyl group

(b) 6-Methyl-1 2-benzanthracene-5 10-dicarboxylic acid (III), arising from fission at the 15–16 bond

or

(c) 1 2-Benzanthracene-5 6 10-tricarboxylic acid (IV), arising from a combination of these
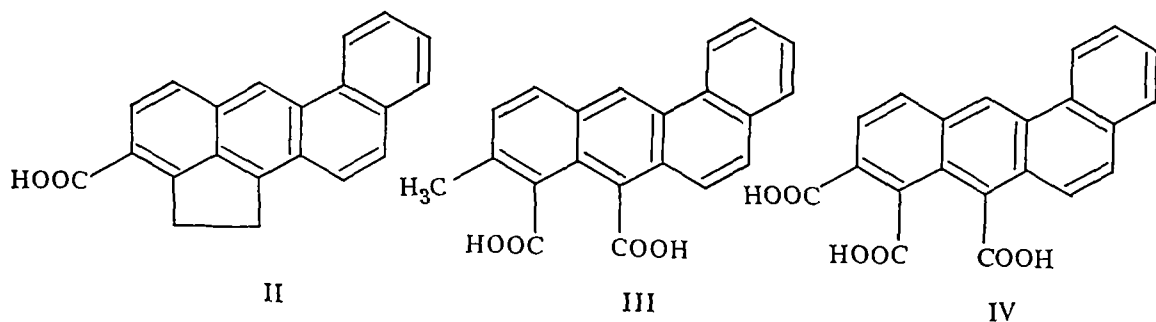
II   III   IV

If the metabolite is either the di-(III) or tri-(IV) carboxylic acid, then anhydride formation is theoretically possible This was tested by heating the compound at 200° C for ten minutes After cooling and dissolving in ethanol no difference in its chromatographic behaviour and absorption spectrum was observed A tentative conclusion therefore is that the metabolite is cholanthrene-20-carboxylic acid (II)

The absence of the free phenol and carboxylic acid from the gall bladder and small intestine indicated their formation from the conjugated metabolites present

in these organs   This was confirmed on enzymatic hydrolysis of the conjugates when the sulphate fraction yielded free phenol alone whilst the glucuronide fraction yielded a mixture of phenol and carboxylic acid   In the latter case advantage was taken of the differential solubilities of the derivatives in sodium bicarbonate to effect a ready separation of the two components from solution in ether   The same behaviour was observed on acid hydrolysis under nitrogen but a further phenomenon recorded in this instance was the liberation of a trace amount of 20-methylcholanthrene from the glucuronide fraction

The absorption spectra of the conjugated fractions, shown in Fig 4, establish that the aromatic benzanthracene nucleus is intact   It is concluded from this work therefore that the X_1 type metabolite is a sulphuric acid ester of a phenolic derivative of 20-methylcholanthrene whilst the X_2 type contains a mixture of glucuronic acid esters of the same phenol and cholanthrene-(20)-carboxylic acid together with an acid-decomposable precursor of the hydrocarbon   Indications of the presence of other water soluble metabolites have also been obtained but these have not been pursued further

(D) 1 2 5 6-Dibenzanthracene

A phenolic derivative, 4' 8'-dihydroxy-1 2 5 6-dibenzanthracene, was the first metabolite of 1 2 5 6-dibenzanthracene to be identified (Cason and Fieser, 1940 , Dobriner, Rhoads and Lavin, 1942)   Subsequent investigation by Heidelberger and his collaborators in the United States established that the metabolism of this hydrocarbon is accompanied by extensive degradation of the aromatic nucleus (Heidelberger and Jones, 1948 , Heidelberger, Kirk and Perkins, 1948) and consistent with this finding is the low yield of fluorescent material which has been isolated in the present work   Owing to the small amounts of metabolites obtained it has not been possible to characterise the compounds individually by absorption spectroscopy although a mixture of the conjugated derivatives extracted from bile possessed absorption indicative of an intact dibenzanthracene nucleus

Enzymatic hydrolysis of the two conjugated fractions as described resulted in the liberation of a phenolic derivative as adjudged by the fluorescence colour change from blue to yellow occurring on the addition of sodium hydroxide   It was concluded from this behaviour therefore that a phenolic derivative, presumably 4' 8'-dihydroxy-dibenzanthracene, is excreted via the bile in conjugation with sulphuric and glucuronic acids   The conjugates could not be detected within the liver or kidney and were absent from the caecum and large intestine   Free phenol was present in the latter organs, however, suggesting that hydrolysis of the conjugates occurs at this site

These findings are supported by the data reported by Heidelberger, Kirk and Perkins (1948) on the excretion of ^{14}C-labelled dibenzanthracene following intravenous injection in the mouse   The extraction procedure adopted by these workers enabled the radioactivity of the material under investigation to be split into four fractions which they considered to contain respectively

(1) Unchanged hydrocarbon
(2) Unconjugated material
(3) Extremely water soluble organic substances together with some less soluble material which was conjugated with water solubilising groups
(4) A solid residue which was partly soluble in water

Analysis of the data obtained respectively from bile and faeces shows that, during passage through the intestine, there is a transfer of radioactivity from fraction 3, the major component of the biliary activity, to fractions 2 and 4 The increase in fraction 2 is most pronounced, from 0 7 per cent in the bile to 14 per cent in the faeces, suggesting that unconjugated derivatives in the faeces are derived from conjugated compounds excreted in the bile

It was further shown by these workers that fraction 2 of the faeces contains a mixture of phenolic, acidic and neutral components so that the same sequence of conjugation and hydrolysis presumably occurs with the dicarboxylic acid degradation products as has been established for cholanthrene-(20)-carboxylic acid in the present work

(E) Anthracene

The metabolism of anthracene was studied by Boyland and Levi (1935, 1936a, 1936b) who reported the excretion in the urine of a perhydroxylated derivative, 1 2-dihydroxy-1 2-dihydro-anthracene, both free and conjugated with glucuronic acid, an unidentified acid-decomposable precursor of the hydrocarbon and 1-anthrylmercapturic acid   The latter has since been shown by Knight and Young (1958) to arise during extraction from the action of mineral acid upon a precursor designated by the general term " premercapturic acid "

The excretion of metabolites in the bile has also been reported (Chalmers and Peacock, 1941 , Chalmers, 1957) but these have not been identified

The present experiments with anthracene have shown that X_1 and X_2 type derivatives, analogous to those yielded by the other hydrocarbons studied in this series, are not formed during the metabolism of this compound   The isolation of such compounds, i e 1-anthryl sulphuric and glucuronic acids, from the bile following injection of 1-anthrol, under the same conditions of extraction, established that failure to detect these derivatives during metabolism of the parent hydrocarbon was not due to defects of the extraction process

The situation was found to be quite different however when the bile and aqueous extracts of the duodenum and small intestine were first subjected to mild acid hydrolysis   Under these conditions an X_2 type derivative, identical with 1-anthrylglucuronic acid, and free anthracene were readily extractable from the acid solutions   A blue fluorescent chloroform soluble fraction was also present but this was not investigated further

As the 2-hydroxy-1 2-dihydro- 1 -anthrylglucuronic acid excreted in the urine was found by Boyland and Levi (1936a) to break down readily under the influence of acid to yield 1-anthrylglucuronic acid, it was concluded from the above behaviour that this perhydroxylated conjugate is also excreted in the bile together with an acid-decomposable precursor of anthracene and possibly a " premercapturic acid "

DISCUSSION

The investigations reported in this and previous publications were part of a programme designed to determine the role, if any, played by the metabolism of polycyclic hydrocarbons in induced carcinogenesis   Should the formation of the metabolites be implicated in the carcinogenic mechanism or themselves be the initiators of it—cf the mode of action of 2-naphthylamine (Bonser, Clayson,

Jull and Pyrah, 1952)—it was considered necessary that the following require-
ments be fulfilled

   (a) They should be present within the tissues of the body where the
carcinogenic effect is applied

   (b) The same type of metabolites should be formed from all carcino-
genic but not non-carcinogenic hydrocarbons (quantitative factors are
ruled out by the fact that the carcinogenic members exert their effect
when present in only trace amounts)

   (c) If themselves the proximate carcinogenic agents then this activity
should be detectable by normal laboratory methods of testing

Requirement (a) was met by the intermediate X_1 and X_2 metabolites of
3 4-benzpyrene previously isolated by Weigert and Mottram (1943)   Extension
of this work to a range of hydrocarbons has now shown that similar intermediates
are formed from the non-carcinogenic pyrene, the weakly carcinogenic chrysene
and 1 2-benzanthracene and the strongly carcinogenic 1 2 5 6-dibenzan-
thracene and 20-methylcholanthrene   These intermediates have been found to
possess a common structure, i e phenolic derivatives conjugated with sulphuric
acid (X_1) and glucuronic acid (X_2), and the association of acid-decomposable
precursors of the hydrocarbons with the X_2 fractions suggests that these too may
contain a glucuronide conjugated hydroxyl group   Indeed, the experiments
with pyrene (Harper, 1957a, 1958a) suggest that, in like manner to the phenolic
conjugates, the glucuronide moiety of the precursors is split off during excretion
for the pyrene precursor in the faeces was then found in association with the
neutral quinone fraction on the chromatogram   Consistent with this behaviour
would be the presence of an α β-dihydro-α-hydroxy configuration similar to
that established for the precursor of naphthalene (Boyland and Solomon, 1955)

The metabolisms of 1 2 5 6-dibenzanthracene and 20-methylcholanthrene
are further complicated by the formation of carboxylic acid derivatives but
this does not appear to be a general feature of the carcinogenic series

The anomalous behaviour of anthracene must be attributed to the exclusive
process of perhydroxylation, as opposed to that of hydroxylation, operating on
this hydrocarbon   As dihydrodiols appear to undergo conjugation exclusively
with glucuronic acid the failure of anthracene to bring about any significant
increase in the level of ethereal sulphate excretion in the urine (Elson, Goulden
and Warren, 1945) is consistent with the finding that phenol formation does not
occur

Only in the case of 3 4-benzpyrene has it been possible to isolate sufficient
of the intermediate metabolites for carcinogenicity testing   These have been
tested singly, in combination with each other and in combination with a co-
carcinogen, croton oil, but in no case has malignant tumour formation been
observed (Harper, 1957b, 1958d)   Other hydrocarbon derivatives tested for
activity include the hypothetical metabolites of anthracene, 1- and 2-anthrol
These were found to be inactive suggesting that the non-carcinogenic nature of
this hydrocarbon is not due to the protective influence of dihydrodiol formation
(unpublished data)

The overall conclusion from this work therefore is that there appears to be no
specific difference in the chemical nature of the metabolites derived respectively
from carcinogenic and non-carcinogenic hydrocarbons   It is unlikely then that

the known metabolites represent the proximate carcinogenic agents and this is supported experimentally both by the negative findings of carcinogenic activity referred to above and by the negative findings reported for phenolic and acidic metabolites by other workers (e g Hartwell, 1951 , Heidelberger and Wiest, 1951 , Allen, Boyland and Watson, 1956)

Conversely, the formation of similar metabolites from both carcinogenic and non-carcinogenic hydrocarbons would appear to provide no indication of an association of metabolism with carcinogenesis   However, one important difference not yet referred to lies in the position of the hydrocarbon molecule at which biochemical hydroxylation takes place   This is exemplified by reference to Table II in which existing data on the chemical reactivities and metabolic hydro-

TABLE II

Hydrocarbon	Reactive positions	Reactive bonds	Metabolic positions (phenols)	Metabolic bonds (dihydrodiols)	Carcinogenic activity
Naphthalene	1 (2)	1–2	1 (2)	1–2	—
Anthracene	9, 10	{ 1–2 3–4 }	(9, 10)	1–2	—
Phenanthrene	9, 10	9–10		9–10 (1–2)	—
Pyrene	3	1–2	3		—
Chrysene	2	1–2	3		+
1 2-Benzanthracene	9, 10	3–4	4′		+
9 10-Dimethyl-1 2 benzanthracene	9, 10	3–4	4′		+
1 2 5 6-Dibenzanthracene	9, 10	{ 3–4 7–8 }	4′, 8′ 2′, 6′ }		+
3 4-Benzpyrene	5 (10)	6–7	8, 10, F$_1$ (5, 5 8, 5 10)		+
20 Methylcholanthrene	15 (11, 14)	6–7	2 or 4? (not 15)		+

Numbers in brackets refer to positions of secondary activity

xylation of a range of hydrocarbons is summarised   The non-carcinogenic members, it will be seen, are characterised by a tendency to undergo either phenol or dihydrodiol formation, the former occurring at the reactive positions of the molecule and the latter at the reactive bonds   The carcinogenic members on the other hand are typified by a tendency to undergo phenol formation only and this occurs, in general, at positions of the molecule which are inert towards chemical attack   In order to account for this fact that carcinogenic hydrocarbons are subjected to hydroxylation in these normally inert positions it has been suggested (Dickens and Weil-Malherbe, 1945 , Boyland, 1948, 1950, Berenblum and Schoental, 1949) that the reactive centres of the carcinogen are initially blocked by cellular constituents and that, in this bound state, the " metabolic positions " become activated   An alternative possibility, however, is that different hydroxylating mechanisms are operative in the metabolism of carcinogenic and non-carcinogenic hydrocarbons   It is hoped to discuss both these possibilities in greater detail in a succeeding paper   At present it is sufficient to say that any association of a known metabolic process with carcinogenesis most probably lies within or before the primary stage of aromatic hydroxylation

SUMMARY

1 Previous studies of the intermediary metabolism of pyrene and 3 4-benzpyrene have been extended to a range of hydrocarbons, namely 1 2-benzanthracene, chrysene, 20-methylcholanthrene, 1 2 5 6-dibenzanthracene and anthracene   All of these, with the exception of anthracene, have been found to undergo hydroxylation to phenolic derivatives   These are excreted mainly via the bile in conjugation with sulphuric and glucuronic acids and hydrolysis then occurs during passage through the caecum and large intestine   Consequently it is the free phenols that are detected when investigations are confined to the faeces alone

2 Acid-decomposable precursors of the hydrocarbons have been detected in association with the glucuronide fraction on the chromatogram   The presence of a glucuronide moiety in the precursors is therefore indicated and experiments with pyrene suggest that, in like manner to phenolic conjugates, this is split off during excretion

3 Evidence has been obtained that 1 2-benzanthracene is subjected to hydroxylation in two positions of the molecule, the 4'- and possibly, by analogy with other hydrocarbons, the 2'-

4 20-Methylcholanthrene has also been found to yield a carboxylic acid derivative, provisionally identified as cholanthrene-20-carboxylic acid, and this undergoes the same sequence of glucuronide conjugation and hydrolysis during excretion as has been established for the phenolic metabolite   The data reported on the metabolism of 1 2 5 6-dibenzanthracene-9 10-^{14}C by Heidelberger, Kirk and Perkins (1948) is considered to be consistent with a similar behaviour for the dicarboxylic acid derivatives yielded by this hydrocarbon

5 It is suggested that the anomalous behaviour of anthracene is due to the exclusive process of perhydroxylation operating on this hydrocarbon   The metabolites excreted in the bile have not been isolated but their behaviour towards mild acid hydrolysis identifies them as 2-hydroxy-1 2-dihydro-1-anthryl glucuronic acid and an acid decomposable precursor of the hydrocarbon

6 It is concluded from these studies that the only important difference between the known metabolisms of carcinogenic and non-carcinogenic hydrocarbons lies in the positions of the molecule at which hydroxylation initially occurs   Consequently any association of metabolism with carcinogenesis most probably lies either within or before this stage

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THE POLYCYCLIC HYDROCARBONS METABOLISM, CELLULAR BINDING AND CARCINOGENESIS

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THE data reported in the preceding communication (Harper, 1959) have led to the conclusion that the only important difference between the metabolisms of carcinogenic and non-carcinogenic hydrocarbons lies in the positions of the molecule at which hydroxylation initially occurs   Two possible explanations of this difference were considered to be

(*a*) That the reactive centres of carcinogenic hydrocarbons are initially blocked by cellular material ,

or

(*b*) that different mechanisms of hydroxylation are operative during the metabolism of carcinogenic and non-carcinogenic hydrocarbons respectively

The mechanism of aromatic hydroxylation is a subject which has received much attention in recent years but so far the investigations have been confined to only two members of the polycyclic hydrocarbon series, namely, naphthalene and 3 4-benzpyrene   Fortunately for the purpose of this discussion, however, these may be regarded as typical members of the non-carcinogenic and carcinogenic hydrocarbons respectively

It was first shown by Mitoma, Posner, Reitz and Udenfriend (1956) that an enzyme system capable of hydroxylating a variety of organic compounds was able to effect the *in vitro* conversion of naphthalene into 1-naphthol (but not 2-naphthol) and a dihydrodiol-like compound which yielded 1-naphthol on acid hydrolysis   The system was located in the microsomal fraction of liver homogenate (but not of brain, kidney, lung and muscle) and required reduced triphosphopyridine nucleotide and oxygen for activity

A similar enzyme system was found by Conny, Miller and Miller (1957) to effect the hydroxylation of 3 4-benzpyrene and the products thus obtained were identical to those yielded during *in vivo* metabolism of the hydrocarbon   The " hydroxylase " was similarly located in the liver microsomes and, although both reduced tri- and diphosphopyridine nucleotides (TPNH and DPNH) and oxygen were required for maximal activity, high activity was supported by TPNH and oxygen alone   The system differed from that of Mitoma *et al* (1956) however in not being inhibited by the metal binding compounds α, α'-dipyridyl and *o*-phenanthroline

Further investigation of the naphthalene hydroxylating system by Booth and Boyland (1957, 1958) confirmed the findings of Mitoma *et al* with respect to the cellular distribution and requirements of the system but it was found

that neither α, α'-dipyridyl nor o-phenanthroline inhibited activity to any great extent Also, in like manner to the benzpyrene hydroxylating system, inhibition was not observed with either cyanide or cysteine but strong inhibition occurred in the presence of p-chloromercuribenzoate suggesting the involvement of sulphydryl groups

On this evidence therefore it appears probable that the same enzyme, or closely related enzymes with the same co-enzyme requirements, are responsible for the hydroxylation of naphthalene, a non-carcinogen, and 3 4-benzpyrene, a potent carcinogen If such is the case it is unlikely that such closely related systems operate by different mechanisms

This brings us back to the alternative possibility therefore, of an initial blocking of the reactive centres of the carcinogen by cellular material The problem in this case is to determine at what region of the molecule such binding is likely to occur, for reference to Table II of the preceding communication (Harper, 1959) reveals the presence of both reactive carbon atoms and reactive bonds although these coincide in certain cases

The possibility of binding across reactive non-adjacent carbon atoms was considered by Dickens and Weil-Malherbe (1946) who compared metabolic hydroxylation with the chemical method used most successfully in the synthesis of certain metabolic phenols, i e sulphonation of the meso-quinone followed by reduction and alkali fusion An attempted synthesis of 8-benzpyrenol starting with the 5 10-quinone was unsuccessful owing to failure at the reduction stage but none the less Dickens and Weil-Malherbe considered that this chemical evidence tallies well with the conception that the most reactive centres of the hydrocarbon molecule are blocked with a cellular constituent and that oxidation then occurs at the most reactive centres remaining

The other possibility of binding at the reactive bond of the hydrocarbon was proposed by Boyland (1948, 1950a) and a similar conclusion concerning the formation of 3-chrysenol from chrysene was arrived at by Berenblum and Schoental (1949) The reactive bond corresponds, in most cases, to the so-called K-region of the molecule and great significance is attached to this proposal when considered in relation to the evidence obtained by the Heidelberger school on the binding of hydrocarbons to protein within the skin In the case of one hydrocarbon, 1 2 5 6-dibenzanthracene, it has actually been established that about 25 per cent of the bound total is attached through both mono- and di-amido linkages in one K-region (Bhargava and Heidelberger, 1956) so that here is experimental evidence in support of the Boyland hypothesis

A theoretical treatment of this problem by Pullman and Pullman (1955) was based upon the somewhat arbitrary assumption that the hydrocarbon, bound through its K-region, then exists in an ortho-quinonoidal configuration In the example selected, 1 2-benzanthracene, it was calculated that the reactive centre of the hydrocarbon bound in this way then resides on the 3' carbon atom and not the metabolic 4' position This difficulty was overcome by postulating the formation of an epoxide across the 3'–4' bond which then undergoes hydrolysis under enzymatic control to yield the 3'–4' dihydrodiol Finally the elements of water are split off leaving the hydroxyl group in the 4' position

Despite the obvious limitations of such a hypothetical treatment it will be seen that both these proposals, one involving a para and the other an ortho form of binding, are unable in themselves to account for the formation of more

than one phenol from the same hydrocarbon   Indeed, the mechanism proposed
by Pullman and Pullman (1955) leads to the formation of the same phenol as
that obtained from the para-quinone by the described chemical synthesis
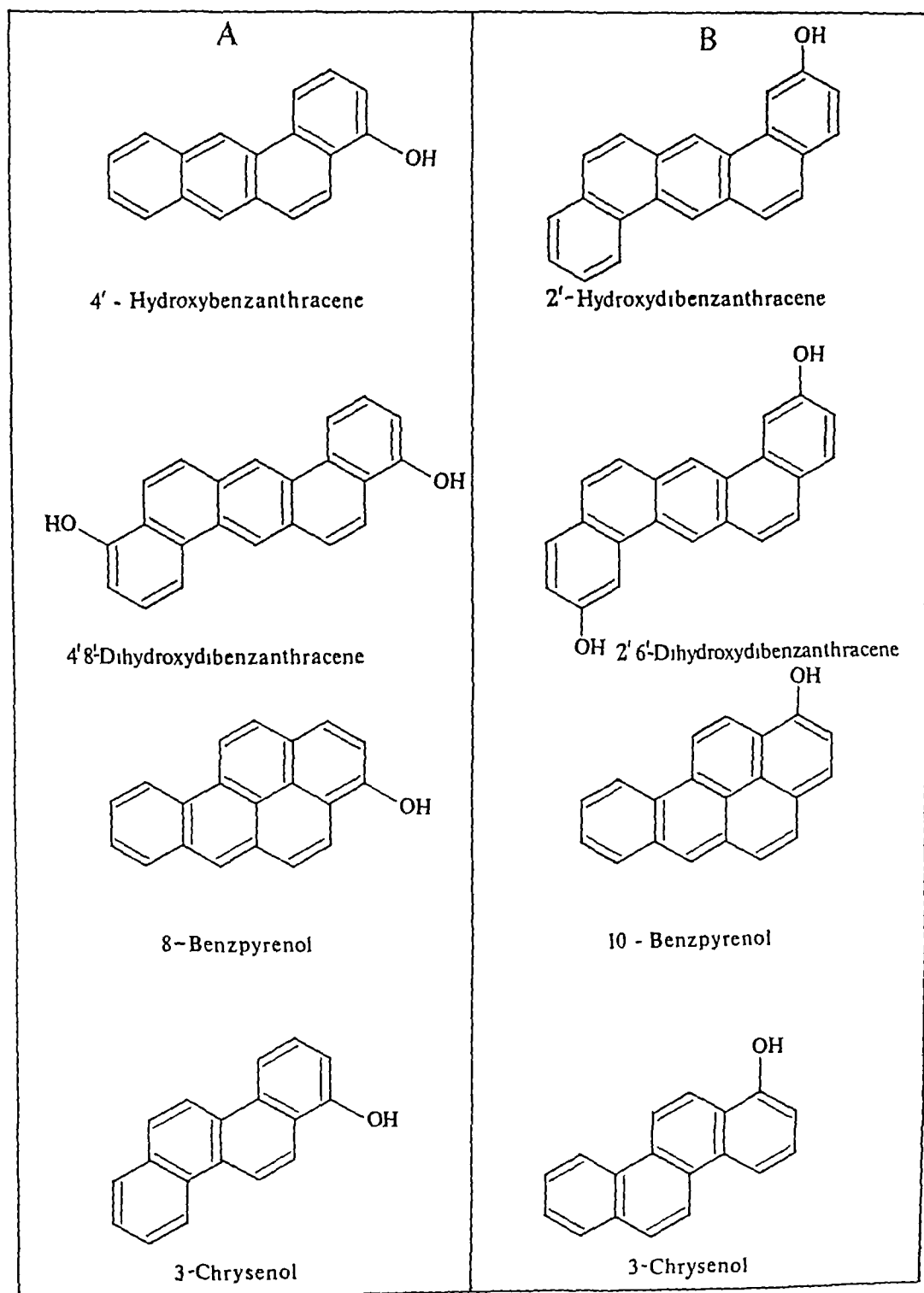Examples of phenolic metabolites are shown in Fig 1 and it will be seen that

A

4' - Hydroxybenzanthracene

B

2'-Hydroxydibenzanthracene

4'8'-Dihydroxydibenzanthracene

2' 6'-Dihydroxydibenzanthracene

8-Benzpyrenol

10 - Benzpyrenol

3-Chrysenol

3-Chrysenol

Fig 1 —Phenolic metabolites of the polycyclic hydrocarbons

these can be classified on the basis of the structural relationship they bear to one another into two distinct groups designated as A and B in the diagram   What is proposed by the author therefore is that the hydrocarbon may undergo both ortho and para forms of binding during hydroxylation and that in these two states different secondary positions of the molecule become activated   Such a proposal is not entirely speculative for only 25 per cent of the total bound 1 2 5 6-dibenzanthracene could be accounted for by binding in the K-region (Bharagava and Heidelberger, 1956)   The remaining 75 per cent is presumably bound at some other region of the molecule   Also 1 2 3 4-dibenzanthracene does not possess an active K-region and yet is bound to an even greater extent than the 1 2 5 6-isomer (Heidelberger and Moldenhauer, 1956)   In this case evidence of binding in the reactive meso-positions has in fact been obtained (Oliverio and Heidelberger, 1958)

The essential problem then is to determine which form of binding is responsible for the formation of Group A phenols and which form for Group B   For this purpose the effect of both forms of binding will be considered in relation to the products yielded by individual hydrocarbons   As the exact mode of binding is as yet unknown the hydrocarbon will be assumed to form an addition type of complex as proposed by Boyland (1950a)   In such compounds the nuclear bond system is that of the ortho- or para-quinone although there is little contribution of the bond linkages to the resonance energy of the system   Should the binding prove to be of a different nature however, it is anticipated that similar considerations will apply

1 2 5 6-Dibenzanthracene

Three phenolic metabolites of this hydrocarbon have now been identified, the 4' 8'-dihydroxy derivative from the rat and mouse (Cason and Fieser, 1940) and the 2'-hydroxy and 2' 6'-dihydroxy derivatives from the rabbit (Labudde and Heidelberger, 1958)   In this instance where the molecule is symmetrical about a central axis, it is to be anticipated that binding across this central axis (I) will assure equal activation in the two halves of the molecule

I

II

III
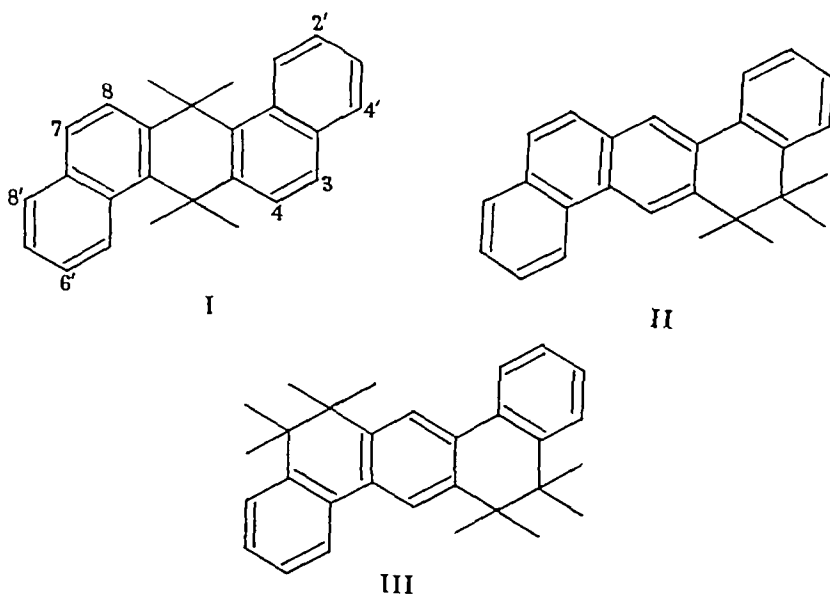
(cf the sulphonation of 1 2 5 6-dibenz-9 10-anthraquinone) Monohydroxylation would not therefore be expected If on the other hand, binding were to occur at one of the two K-regions (II) then in this instance, where the activating group is asymmetrically situated, it is unlikely that two positions of the molecule would become activated to the same extent Consequently the formation of a monohydroxylated derivative only would be favoured In the event of binding at both K-regions, however, the activation in both halves of the molecule would then be equal and dihydroxylation would most probably take place

It will be seen therefore that the formation of both mono- and di-hydroxylated products in the rabbit is best explained on the basis of an ortho form of binding at one and both K-regions respectively Conversely the formation of a di-hydroxylated—but not a monohydroxylated—derivative in the rat and mouse is consistent with a para form of binding across the reactive meso-positions The inference is therefore that species differences in metabolism are due to differences in the region of the molecule at which linkage to cellular material occurs during hydroxylation in these species If this reasoning is correct an ortho form of binding would appear to be favoured in the rabbit and a para form in the rat and mouse The question immediately arises, however, as to why the mouse does not yield a mixture of phenolic metabolites for K-region binding in this species is now an experimentally established fact One obvious answer is that the 2'- and 2' 6'-hydroxylated compounds are formed but in amounts so small that they have so far escaped detection This behaviour would then fall into line with that of 3 4-benzpyrene for which only a quantitative difference in metabolism has been reported (see later) A possible explanation of this is that the bound complex II behaves as a 2 3-disubstituted phenanthrene derivative and as such is particularly susceptible to oxidation at the reactive 9–10 bond, corresponding to the 7–8 bond of dibenzanthracene Consistent with this hypothesis is the formation within the tissues of the 3 4-quinone (Heidelberger, Hadler and Wolf, 1953) and 2-phenylphenanthrene-3 2'-dicarboxylic acid (Bhargava, Hadler and Heidelberger, 1955)

1 2-*Benzanthracene*

IV   V
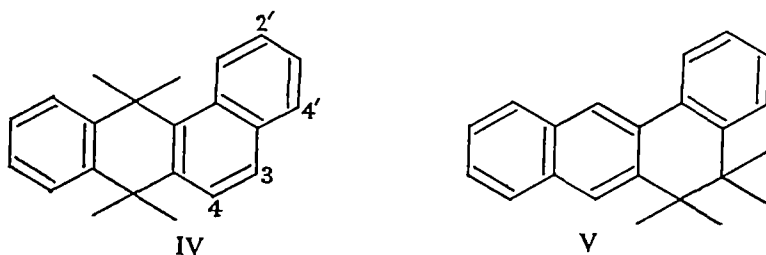
Application of this reasoning to 1 2-benzanthracene leads to the conclusion that, by analogy, the 4'-hydroxy metabolite, a Group A phenol, is formed as a result of binding across the reactive meso-positions (IV) The formation of 2'-hydroxy-1 2-benzanthracene is therefore to be anticipated as a consequence of binding in the K-region (V) and it is significant that the evidence reported in the preceding publication is consistent with the formation of an additional phenolic metabolite It would obviously be of great interest to determine the major site of metabolism in the rabbit where, according to theory, a K-region

binding of hydroxylation is favoured   Investigations are already in hand to test this proposal
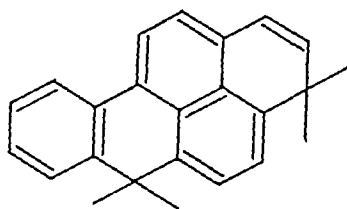
3   4-Benzpyrene

The formation of six different phenolic metabolites from 3   4-benzpyrene has now been reported, 5-benzpyrenol (Pihar and Spáleneý, 1956), 8-benzpyrenol (Berenblum, Crowfoot, Holiday and Schoental, 1943), 10-benzpyrenol (Berenblum and Schoental, 1946), 5   8- and 5   10-dihydroxy-benzpyrenes (Conney et al, 1957) and an unidentified phenol designated as the F_1 metabolite (Weigert and Mottram, 1946 , Harper, 1958)
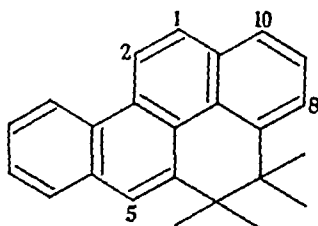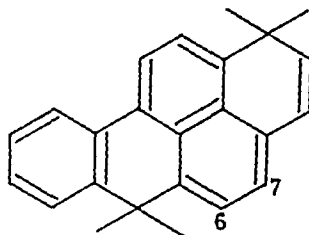
VI   VII

VIII

The ease with which 3   4-benzpyrene undergoes oxidation to a mixture of 5   8-, 5   10-, and possibly 6   7-quinones (Cook and Schoental, 1950) suggests that the molecule readily assumes the nuclear bond structures VI–VIII   It is possible then that binding may occur across the 5   8-, 5   10-, and 6   7- (K-region) positions of the molecule, each of which leads to the formation of a different phenol   By analogy with 1   2   5   6-dibenzanthracene an ortho form of binding in the K-region (VI) may be expected to activate the 10-position and it is significant that 10-benzpyrenol, a Group B phenol, is formed to a greater extent in the rabbit than in the rat and mouse (Berenblum and Schoental, 1946)

As the para configuration of structure VII is analogous to that of a true meso-quinone it is then possible that binding across the 5,10 positions would result in activation of the 8-position (Group A)   By elimination, formation of the F_1 metabolite may then be attributed to binding in the 5,8 positions

An alternative treatment, however, is to regard the K-region bound structure VI as a 6   7-disubstituted chrysene   On this interpretation position 2 of the chrysene molecule, corresponding to the 1-position of benzpyrene, would be expected to be most active and indeed may be expected to represent a major site of hydroxylation   It is possible therefore that the F_1 derivative is 1-benzpyrenol and consistent with this hypothesis is the fact that F_1 is undoubtedly

51

the major product of hydroxylation during the first few hours following injection of 3 4-benzpyrene (Weigert and Mottram, 1946, Harper, 1958)

If structure VI does in fact exhibit the normal chemical reactivity of chrysene a further stage of binding may occur at the reactive 1–2 bond (IX) and, by analogy with chrysene (see later), lead to the formation of 10-benzpyrenol
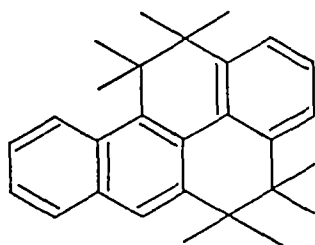
IX

The other phenolic metabolites of benzpyrene, 5-benzpyrenol and the 5 8- and 5 10-dihydroxy derivatives, differ from the others so far considered in that hydroxyl groups have entered the molecule in the chemically reactive positions A possible explanation of this is that hydroxylation may occur to a certain extent when the hydrocarbon is in an unbound state   Perhaps significantly the latter compounds have only been detected in *in vitro* studies with the isolated micro-somal system in which the normal *in vivo* pattern of cellular binding may not prevail to the same extent

Chrysene

The hydroxylation of chrysene is more difficult to interpret for the 3-chrysenol rat metabolite (Berenblum and Schoental, 1949) may be regarded as either a Group A or Group B phenol   By analogy with other hydrocarbons an ortho form of binding in the K-region (X) must be favoured in this instance   A para form of binding across the reactive 2,8 positions (XI) on the other hand may be expected to give rise to a symmetrical dihydroxylated derivative
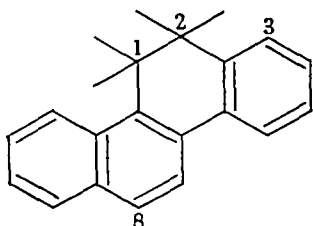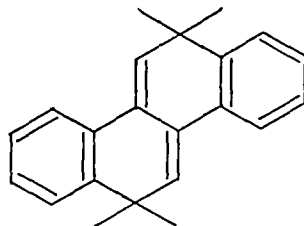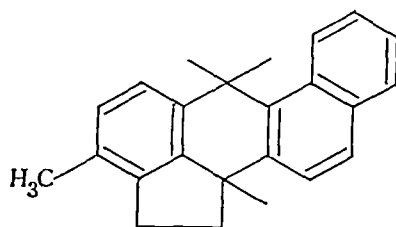
X   XI

20-Methylcholanthrene
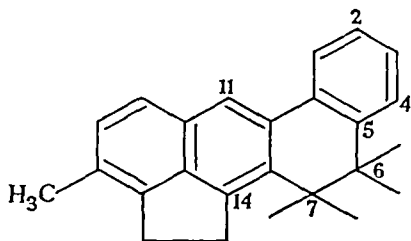
XII   XIII

The two possible modes of binding for 20-methylcholanthrene are shown in XII and XIII By analogy with other hydrocarbons these may be expected to activate the 2 and 4 positions respectively The ortho bound structure, XII, however, may be regarded as a disubstituted acenaphthene derivative and as such would be more prone to oxidation at the 15–16 bond of the pentacyclic ring (cf the metabolism of acenaphthene, Chang and Young, 1943) The evidence reported in the preceding paper suggests that this does not occur to any appreciable extent and a para form of binding (XIII) across the 11, 14 positions is therefore favoured A possible structure for the phenolic metabolite isolated in that work is therefore 4-hydroxy-20-methylcholanthrene

So far these theoretical considerations have been confined to the carcinogenic members of the polycyclic hydrocarbons As was pointed out in the preceding publication (Harper, 1959) the products obtained from non-carcinogenic hydrocarbons are consistent with the view that hydroxylation occurs at the most reactive positions of the molecule and perhydroxylation at the most reactive bonds although the perhydroxylation of phenanthrene at the 1–2 bond in the rabbit is an obvious exception to this generalisation This behaviour may be interpreted as indicating that the non-carcinogenic members are metabolised primarily when in an unbound state and would be in accordance with the conclusion of Heidelberger and Moldenhauer (1956) that non-carcinogenic hydrocarbons do not undergo binding to protein to any appreciable extent The evidence of Hadler, Darchun and Lee (1957), however, would suggest that the non-carcinogens may go through a transient protein-bound phase of at least the same order of magnitude as that observed with the carcinogens The findings of Calcutt (1958) are not only consistent with this view but also show that binding may occur in other tissues apart from the skin It is of interest therefore to speculate upon the effects of ortho and para forms of binding on the reactivities of this class of compounds

Naphthalene
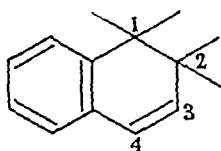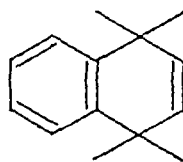

XIV   XV

A variety of hydroxylated products, 1- and 2-naphthol (Bourne and Young, 1934 , Corner and Young, 1954, 1955), 1 2-dihydroxy-naphthalene (Corner and Young, 1954, 1955), 1 2-dihydroxy-1 2-dihydro-naphthalene (Young, 1947) and 1-hydroxy-1 2-dihydro-naphthalene (Boyland and Solomon, 1955) are formed during the metabolism of this hydrocarbon In all cases, however, attack is at the 1–2 bond and any form of binding must therefore activate this region of the molecule

The two possible modes of binding for naphthalene are shown in XIV and XV It is highly improbable that either of these lead to activation in the unsaturated benzene ring and the only possible means of activation of the metabolic bond is therefore via the ortho-bound structure XIV, as proposed by Boyland (1950b)

Anthracene

<div style="text-align:center">9 1 2 3 10 4</div>
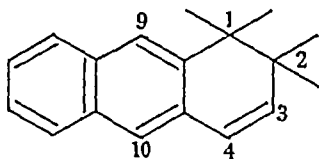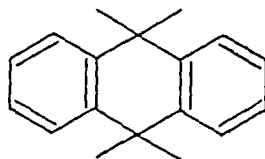
XVI   XVII

Unlike naphthalene the reactive carbon atoms of anthracene do not coincide with the reactive bond   If, as concluded earlier, hydroxylation of the unbound hydrocarbon occurs at the reactive positions of the molecule then the derivative 9 10-dihydroxyanthracene (or 9 10-anthraquinone) is to be anticipated as a result of this process   There is in fact evidence that the 9 10-quinone is excreted as a metabolite of anthracene in the urine (Boyland and Levi, 1936) although the possibility of this being present as an impurity in the administered hydrocarbon was not discounted in that work   Since then however it has been reported that administered 9 10-anthraquinone is itself subjected to hydroxylation (Sato, Fukuyama, Yamada and Suzuki, 1956) so that the quinone isolated by Boyland and Levi was probably a true product of hydroxylation and not due to impurity   The major point of attack, however, is at the 1–2 bond resulting in the formation of 1 2-dihydroxy-1 2-dihydro-anthracene (Boyland and Levi, 1935)   What must be decided, therefore, is whether the ortho or para bound structures (XVI and XVII) above are characterised by any marked reactivity at the 1–2 bond

Unfortunately little is known of the reactivity of the 1,2 addition compounds although the fact that the perhydroxylation of anthracene with Criegie's reagent (Cook and Schoental, 1948) leads to the formation of the 1 2 3 4-tetrahydroxy-1 2 3 4-tetrahydro derivative (XVIII) and not the 1 2-dihydrodiol (XIX) suggests that marked activation of the 3–4 bond may occur, assuming the course of the reaction to be as shown
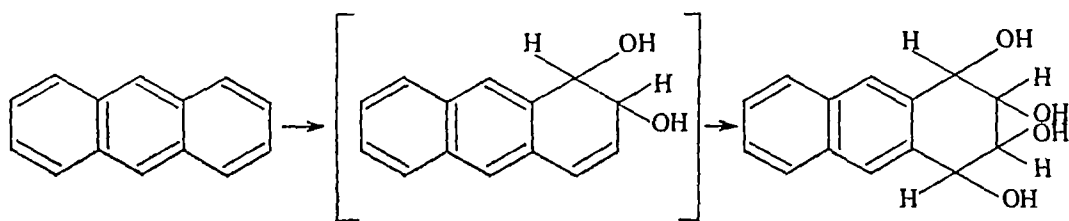
XIX   XVIII

The process of para-blocking in the 9,10 position, however, as with hydrogenation and quinone formation, leads to the formation of particularly stable compounds in which the reactivity of the molecule as a whole is greatly reduced by comparison with that of anthracene   Addition at these points therefore is to be regarded as a deactivating rather than activating mechanism

An ortho form of binding (XVI), as proposed by Boyland and Wolf (1950), is therefore favoured in this instance although the alternative para form is not entirely discounted

Phenanthrene

Phenanthrene is the sole non-carcinogenic hydrocarbon for which a species difference in the site of hydroxylation has been reported, the 9 10-dihydrodiol being formed in the rat and mouse and a mixture of the 9 10- and 1 2-dihydrodiols in the rabbit (Boyland and Wolf, 1948, 1950) The fact that the carbon atoms comprising the highly reactive 9–10 bond are themselves the reactive positions makes it unlikely that perhydroxylation at this bond is due to binding at some other region of the molecule
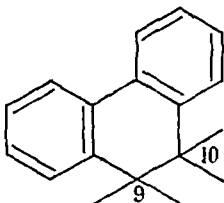
XX

The additional formation of the 1 2-dihydrodiol in the rabbit, however, may be attributed to binding across the reactive 9–10 bond (XX), as proposed by Boyland and Wolf (1950), and provides further support for the suggestion that an ortho form of binding is favoured in the rabbit The fact that 9 10-dihydrophenanthrene undergoes Friedel and Crafts' acylation exclusively in the 2-position is evidence that such addition may have a directive influence

To summarise these proposals, therefore, it can be said that the metabolism of non-carcinogenic hydrocarbons is best explained on the assumption that hydroxylation occurs when they are either in a free state or bound to cellular material through their most reactive bond The metabolism of carcinogenic members on the other hand would suggest that hydroxylation occurs principally after binding and the formation of different phenols from the same hydrocarbon is then explainable if it is postulated that binding may occur either at the reactive bond or reactive positions of the molecule On such reasoning it is tentatively concluded that species differences in the site of hydroxylation are due to differences in the relative contributions of these distinct forms of binding to the total amount bound in any one species An ortho form of binding at the reactive bond would appear to be favoured in the rabbit and a para form across the reactive positions in the rat and mouse

A critical test of these proposals is the verification that the reactive centres of the ortho and para addition products, or perhaps those of the derived ortho- and para-quinonoidal configurations, do in fact correspond to those attacked during metabolism Furthermore, as the binding data referred to in this discussion has been obtained from skin, not only must it be established that the same system of microsomal hydroxylation is operative within this tissue but also that the hydrocarbon is actually subjected to binding during hydroxylation in the system There is evidence in fact that the latter condition is attained, for it was found by Conney *et al* (1957) that mild alkaline hydrolysis (methanolic potassium hydroxide at 3° C for 24 to 48 hours) was necessary for the quantitative liberation of 3 4-benzpyrene after addition to the *in vitro* hydroxylating system Further investigation is obviously required to establish this point and

other limitations will doubtless be exposed when metabolic studies are extended to other hydrocarbons and other species   The proposals are not put forward in any dogmatic sense, therefore, but as a possible explanation of certain known facts concerning the biochemical hydroxylation of polycyclic hydrocarbons, an explanation which may be checked by direct experimentation

The final question to be considered is whether these theoretical proposals provide any indication that the process of hydroxylation is associated with the carcinogenic response elicited by certain hydrocarbons   If the non-carcinogenic members of this series do in fact undergo hydroxylation when in an unbound state then an obvious conclusion is that it is the binding, either ortho or para, of the carcinogens which is a contributing factor in carcinogenesis   If, however, the non-carcinogens are first subjected to binding prior to hydroxylation then, as theoretical considerations suggest that this occurs at the reactive bond rather than across the reactive positions, this would appear to exclude the ortho form of binding from the carcinogenic mechanism   Furthermore, the proposal that species differences in metabolism between the rabbit and rat and mouse respectively may be attributed to the greater contribution of ortho binding in the former species is inversely paralleled by the susceptibility of these species towards the induction of carcinogenesis by the hydrocarbons   1 2 5 6-Dibenzanthracene, for example, is a potent carcinogen for the rat and mouse but is without activity in the rabbit   If the theory is correct, therefore, there would appear to be no association between the ortho or K-region binding of hydroxylation and carcinogenesis   It is tempting to speculate then that the para form of binding may be an important factor governing the carcinogenic response and certain evidence may be cited in support of this view   Thus the blocking of the para-positions in compounds such as cis-9 10-dimethyl-9 10-dihydro-1 2 5 6-dibenzanthracene and 9 10-dimethyl-1 2-benzanthracene-α, β-endo-succine acid is accompanied by a marked reduction in the carcinogenic potencies of the parent hydrocarbons from which these are derived   Such behaviour may be interpreted as an indication that substitution in the meso-positions of these hydrocarbons prevents the process of para-binding during hydroxylation   Also, 9 10-dimethyl-1 2-benzanthracene is considerably more carcinogenic than is the parent 1 2-benzanthracene and this behaviour is reflected in the reactivities of these compounds towards the para-addition of maleic anhydride, this occurring more readily with the dimethyl derivative (Bachmann and Chemerda, 1938 , Newman and Otsuka, 1959)   In other words the effect of methyl substitution in the 9 10-positions of 1 2-benzanthracene, and indeed in those of anthracene, is to facilitate the process of para-addition at these points   In this case, however, we are dealing with a specific type of cyclic dienophile addition and it is not surprising therefore that the analogy breaks down when applied to the whole range of polycyclic hydrocarbons   What is envisaged biologically rather is an addition type of complex arising from combination of the carcinogen either with two molecules or with distal groups of the same molecule so that it constitutes in effect a cross linkage between the two   As was stated earlier the exact mode of linkage is as yet unknown   Boyland (1950a) has suggested a simple covalent type of addition whilst Pullman and Pullman (1955) prefer the quinonoidal type of linkage   A third type of addition not yet considered, however, is that of co-ordination   The latter phenomenon was investigated by Kofahl and Lucas (1954) who reported a fair degree of correlation between the carcinogenic

potencies of aromatic hydrocarbons and their co-ordination activity towards silver ion in the argentation reaction   Complex formation with iodine ions has also been observed (Benesi and Hildebrand, 1948) and the solubilising effect of purine derivatives upon the polycyclic hydrocarbons (Weil-Malherbe, 1946) is now well known   Is it not possible therefore that similar co-ordination complexes may be formed between the hydrocarbon and say the ionised groups of an enzyme within the microsomes?   The process of hydroxylation may then be regarded as a neutralisation of the electronic charge resident on the hydrocarbon portion of the complex resulting in the formation of a phenol and consequent liberation of the enzyme

If, as the evidence suggests, the mechanism of hydroxylation is associated with the carcinogenic mechanism, then the origin of this cellular malformation must reside within the microsomes   Furthermore, as the hydroxylating activity of the microsomes was found by Conney et al (1957) to remain unchanged after preincubation with ribonuclease, the field of action may be narrowed down still further to the non-ribonucleic acid fraction of these organelles   It is significant therefore that Fiala and Fiala (1959) have concluded on entirely different grounds that the non-ribonucleoprotein fraction of the ergastoplasm is the origin of the hepatic carcinogenic response elicited by azo dyes

The suggestion, however, that there may be an association between a para form of binding and carcinogenesis is in conflict with certain experimental evidence on this subject

The hydrocarbon 1 2 3 4-dibenzanthracene for example, does not possess a K-region as such and yet undergoes extensive binding to protein within the skin (Heidelberger and Moldenhauer, 1956)   The logical explanation of this behaviour is that binding occurs at the reactive meso-positions of the molecule and yet this hydrocarbon is, at most, a very weak carcinogen   Also, the evidence reported by Oliverio and Heidelberger (1958) in support of a relationship between K-region binding and carcinogenic activity in the 1 2 5 6-dibenzanthracene series is most convincing although not without certain anomalies

What is possible, however, is that we are dealing with two different and possibly independent processes, one concerned with overall binding to cellular protein and the other with binding, possibly to enzymes, within the microsomes where the hydroxylating system is active   There is in fact evidence that two such forms of binding may exist, for it was found by Miller (1951) that, although normal alkaline hydrolysis was effective in liberating a fluorescent acidic derivative from the precipitated protein of skin following treatment with 3 4-benzpyrene, a further fluorescent neutral fraction was only released by this procedure when zinc dust was present in the mixture   Significantly the former conditions are also those necessary for the quantitative liberation of 3 4-benzpyrene from the microsomal system of hydroxylation (Conney et al , 1957)

On existing evidence the K-region binding theory of carcinogenesis must obviously be favoured for this phenomenon has been demonstrated within the skin where tumour formation occurs   The microsomal system of hydroxylation on the other hand has so far been detected only within the liver where the induction of hepatoma formation by polycyclic aromatic hydrocarbons is open to doubt   More must be known therefore of the enzymatic system of hydroxylation within the skin and the mechanisms involved before this apparent conflict can be resolved   It is then possible that studies of binding in the in vitro systems may

provide a truer picture of *in vivo* behaviour for, as the hydrocarbon is not added until the processes of cellular disintegration and centrifugation have been carried out, the objections raised by Hadler, Darchun and Lee (1959) no longer apply

Except where stated the chemical data referred to in this work are taken either from Fieser and Fieser (1944), Gilman's ' Organic Chemistry ' (1947) or Elsevier's ' Encyclopaedia of Organic Chemistry ', Supplementary Volume 14 (1951) Data of carcinogenic activity are taken from Hartwell (1951)

SUMMARY

A theory involving linkage to cellular material, possibly enzymes, within the microsomes has been developed to account for the fact that carcinogenic hydrocarbons undergo hydroxylation principally at positions of the molecule which are inert to chemical attack It is postulated that binding may occur either across non-adjacent reactive positions or at the reactive bond each of which leads to activation at different sites of the molecule On this reasoning it is concluded that species differences in the site of hydroxylation are due to differences in the relative contributions of these distinct forms of binding to the total amount bound in any one species An ortho form of binding would appear to be favoured in the rabbit and a para form in the rat and mouse

The hydroxylation of non-carcinogenic hydrocarbons on the other hand is best explained either on the basis of non-binding or of binding through the most reactive bond There would thus appear to be no association between an ortho form of binding during hydroxylation and carcinogenesis It follows therefore that a para form of binding may be of importance in this respect and this possibility is discussed in relation to current views on protein binding and carcinogenesis

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METABOLISM OF 1 2-BENZANTHRACENE IN THE RABBIT

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THE metabolism of 1 2-benzanthracene in rats and mice was investigated by Berenblum and Schoental (1943) who reported the excretion in the faeces of a phenolic derivative identified as 4'-hydroxybenzanthracene by methylation and spectroscopic comparison with known methoxybenzanthracenes Recent studies by the author (Harper, 1959a) confirmed this identification for the mouse faecal metabolite but the spectral analyses were considered to be consistent with the presence of an additional phenolic derivative The latter compound did in fact appear to be the major component of the initial phenolic fraction conjugated with sulphuric acid in the bile It was suggested, by analogy with other hydrocarbons, that this may be the 2'-hydroxy derivative and theoretical considerations (Harper, 1959b) led to the proposal that the formation of this metabolite would be favoured in the rabbit Samples of both 2'-hydroxy- and 2'-methoxybenzanthracene have now been made available through the kind generosities of Professor Badger in Australia and Dr Schoental in this country so that it has been possible to test these proposals by experiment

MATERIALS AND METHODS

The two rabbits used in this work were maintained on a green-free diet for two weeks prior to injection in order to reduce the chlorophyll content of the gut and faeces

Purified 1 2-benzanthracene was prepared for injection either as a colloid in distilled water (1 mg per c c) or as a supersaturated solution in warm tricaprylin (20 mg per c c)

In the first experiment (Experiment A) one rabbit was injected intraperitoneally with 10 c c of the tricaprylin solution and the faeces were collected daily for a period of one week Extraction into xylene via acetone was carried out immediately and the phenolic metabolite was isolated from the pooled xylenes as described previously (Harper, 1959a)

In the second experiment (Experiment B) the remaining rabbit was injected intravenously via the ear vein with 20 c c of colloidal hydrocarbon and killed at 2 hours No fluorescence attributable to the hydrocarbon or its metabolites was discernible within the tissues and the combined gall bladder and small intestine were therefore homogenised and subjected to hot acid hydrolysis (1 N hydrochloric acid at 100° C for 1 hour under nitrogen) Extraction with xylene then removed a blue-fluorescent derivative which was isolated by the procedure referred to above for the faecal metabolite

Experiment A

A solution of the metabolite in ethanol possessed a bright blue fluorescence which changed immediately to yellow on addition of sodium hydroxide Absorption spectroscopy in hexane revealed the presence of absorbing impurity in the shorter wave length region but above 280 mμ the bands recorded (Fig 1, Table I) were, with one exception (see DISCUSSION), almost identical with those of synthetic 2'-hydroxybenzanthracene

TABLE I

Absorption bands in hexane (mμ)

Rabbit faecal metabolite	2'–OH	Mouse metabolite BA–OH	Methylated rabbit metabolite	2'–OMe
—	226–227	226	226–227	227
—	[255–256]	253	[254–256]	[254–256]
—	262	[262]	262–263	262–263
—	269–270	[270–272]	270	269
—	[278–280]	[278–280]	278–280	280
286	286	286	287	287
297	297	297	297	297 5
322–324	325 5	[320–324]	324–326	326–327
337	—	337	—	—
—	341	[340–341]	338–342	340–342
354	354	354–355	354	354 5
373	373	372	372	372
394	394	394	393	393

2'–OH = 2'-hydroxybenzanthracene
2'–OMe = 2' methoxybenzanthracene
BA–OH = free phenolic component of the mouse sulphate conjugate

Methylation of the metabolite with dimethyl sulphate and excess sodium hydroxide yielded a neutral derivative, purified by chromatography on alumina from benzene followed by alumina from cyclohexane The absorption spectrum of this compound proved to be almost identical with that of 2'-methoxybenzanthracene (Fig 2, Table I) and the phenolic derivative excreted in the faeces was therefore concluded to be 2'-hydroxybenzanthracene

Also included for comparison in the above data (Fig 1, Table I) is the absorption spectrum of the phenolic fraction obtained from the sulphate conjugate of mouse bile (Harper, 1959a) This again is very similar to that of the 2'-hydroxy derivative but differs from it in the sharpness of the major band at 286 mμ In this respect it is similar to 4'-hydroxybenzanthracene and a probable explanation of this behaviour is that a mixture of the 2'- and 4'-hydroxy compounds is present, with the former in excess

Experiment B

The primary aim of this experiment, to isolate conjugated metabolites from the gall bladder and small intestine, was prevented by the large amount of acidic colouring matter present in these organs Recourse was had therefore to the hydrolytic procedure described under MATERIALS AND METHODS in the hope
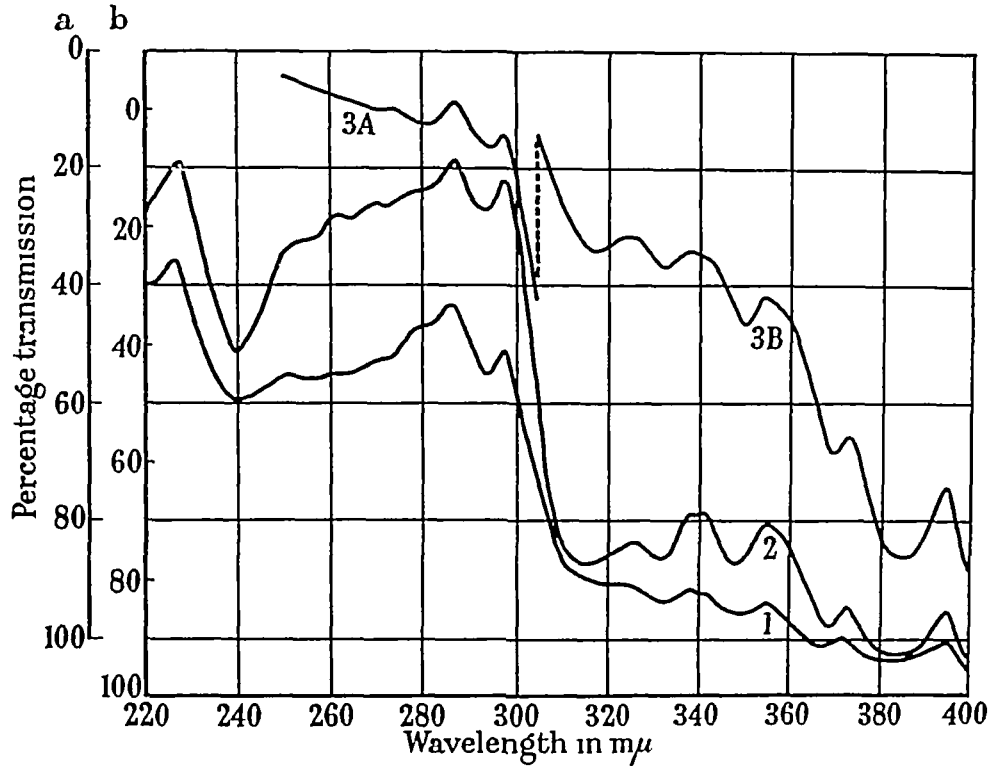
Fig 1 —Absorption spectra in hexane

1 Phenolic component of the mouse sulphate conjugate (b ordinates)
2 2' Hydroxy-1 2 benzanthracene (b ordinates)
3A and 3B —Weak and concentrated solutions of the phenolic metabolite of benzanthracene from rabbit faeces (3A—a ordinates , 3B—b ordinates)
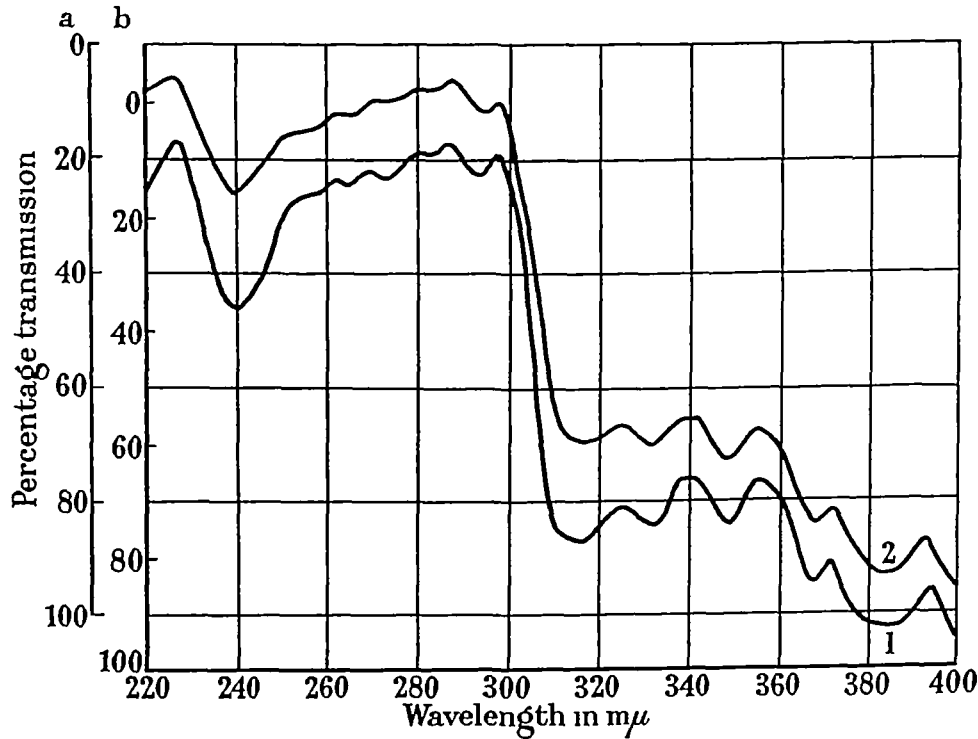

Fig 2 —Absorption spectra in hexane

1 2'-Methoxy-1 2 benzanthracene (b ordinates)
2 Methylated phenolic metabolite of benzanthracene from rabbit faeces (a ordinates)

that a phenolic derivative liberated by this means from a sulphate conjugate would be more amenable to isolation and purification In this respect the experiment was a success and the phenolic fraction thus obtained, although impure, possessed absorption bands identical with those of the faecal metabolite.

DISCUSSION

Although there can be little doubt that the rabbit metabolite isolated in this work is 2'-hydroxy-benzanthracene it is perhaps surprising, in view of the close correspondence existing between other bands, that the rather diffuse 341 mμ band of the synthetic compound should appear at 337 mμ in the metabolite This divergence, however, is undoubtedly due to the presence of absorbing impurity for, as absorption in a mixture is additive, the superimposed effect of decreasing background absorption upon a broad, almost horizontal maximum of this nature will be to decrease absorption in the band passing from shorter to longer wave lengths The overall effect therefore will be a sharpening and hypsochromic displacement of the maximum Significantly, this effect was not observed with the purer methylated form of the metabolite

The isolation of free 2'-hydroxybenzanthracene from the gall bladder and small intestine following acid hydrolysis is consistent with the presence in these organs of a sulphate conjugate, if not perhaps the glucuronide, of the phenol The situation in respect therefore is probably similar to that occurring in the mouse but differs from it in the fact that hydroxylation occurs exclusively in the 2'-position The metabolism of 1 2-benzanthracene in the mouse is indeed analogous to that of 3 4-benzpyrene for, although hydroxylation occurs at more than one point of the molecule, the quantitative relationship existing between the resultant products appears to be time dependent Thus the initial major products yielded by these two hydrocarbons in vivo are probably 2'-hydroxy-benzanthracene and the unidentified F$_1$ metabolite of 3 4-benzpyrene but at later time intervals these are replaced by the structurally analogous 4'-hydroxy-benzanthracene and 8-hydroxybenzpyrene It would appear therefore that the initial products of hydroxylation in the mouse are themselves inhibitors of their own synthesis and herein may lie the secret both of species differences in the site of hydroxylation and in the graded carcinogenic response elicited by the hydrocarbons in these species

In conclusion it can be said that the hydroxylation of 1 2-benzanthracene in the 2'-position and the lack of carcinogenic activity in the rabbit is consistent with the proposal advanced previously (Harper, 1959b) that an ortho form of binding is favoured during hydroxylation in the microsomes of this species The carcinogenic action of this hydrocarbon in the mouse is then to be envisaged as a secondary effect arising from an initial " poisoning " of the defensive ortho-mechanism which then allows the active para form to come into play Similar considerations may also apply in the case of 1 2 5 6-dibenzanthracene (without activity in the rabbit) and 3 4-benzpyrene (reduced activity in the rabbit)

Data of carcinogenic activity are taken from Hartwell (1951)

SUMMARY

1 1 2-Benzanthracene has been found to undergo hydroxylation in the 2'-position after intraperitoneal and intravenous injection in the rabbit The

phenolic metabolite, 2'-hydroxybenzanthracene, is excreted in the faeces although it is probably eliminated initially via the bile in conjugated form

2 Previous data obtained on the metabolism of 1 2-benzanthracene in mice have been re-examined and are consistent with the view that hydroxylation occurs in both the 2'- and 4'-positions with the former predominating during the initial stages of metabolism

3 These results are discussed in relation to former proposals concerning hydroxylation and carcinogenesis

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CONCERNING THE ORIGIN OF SUBSTITUENTS IN POLAR-BOUND DERIVATIVES OF 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE

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FOLLOWING the demonstration by Miller and Miller (1947) of the binding of carcinogenic aminoazo dyes to proteins of the target organ, much work has been directed towards elucidating the reactive sites of both carcinogen and protein

Miller and Miller (1953) have suggested that the linkage between dye and protein is stable to treatment with hot ethanolic KOH Under these conditions hydrolysis of protein occurs so that the recovered polar dye should have an amino acid residue attached To date, insufficient quantities of this polar derivative have been obtained to enable chemical identification of this residue

Gelboin, Miller and Miller (1958) have reported that, a few hours after the intraperitoneal administration of a large dose of 3'-methyl-4-dimethylaminoazo-benzene (3'MeDAB), protein-bound derivatives are detectable in the liver The electrophoretic and spectral properties of the bound dyes obtained, either by prolonged feeding or following a single dose, were similar The findings of Gelboin, Miller and Miller (1958) support the view that a large percentage of the bound dye is bound to certain liver proteins during their synthesis rather than by becoming attached to protein already formed This interpretation is in disagreement with that of Hultin (1956) who suggested that the formation of protein bound dye is independent of protein synthesis

To date, little evidence is available concerning the actual site(s) involved in the protein(s) concerned Kusama and Terayama (1957), Terayama and Kusama (1957), Kusama, Terayama and Teruya (1958) and Terayama, Kusama, Teruya, Kuroda and Nakayama (1958), as a result of spectroscopic studies of synthetic polar-like dyes, have suggested that a tyrosine-aminoazobenzene combination is the most probable model for the polar dye

In this communication the results of an investigation, using isotopically labelled amino acids in an endeavour to determine the origin of substituents in the polar derivative(s) of aminoazo dyes, are presented

METHODS

Care of animals

As a locally available strain of Wistar rats had previously been found to give higher levels of protein-bound dye than rats of Sprague-Dawley strain, the former were used

Three of four rats, weighing 18–31 g were removed from their mother and kept warm for five hours They were then injected intraperitoneally with 0 5 ml of a

2 per cent solution of 3'MeDAB in olive oil and returned to the mother after the remaining members of the litter had been removed   After a further hour the young rats were inspected and ones selected were injected intraperitoneally with ^{14}C labelled amino acid *   A dose of 0 01 mc of amino acid was given when the L isomer was used while 0 020–0 025 mc was given when DL amino acids were employed   The following amino acids were used   DL Alanine-1-^{14}C, L Arginine-^{14}C (G), L Aspartic acid-^{14}C (G), L Glutamic acid-^{14}C (G), Glycine-1-^{14}C, L Histidine-2-(ring)-^{14}C, *Iso*-Leucine-^{14}C (G), DL Leucine-1-^{14}C, L Lysine-^{14}C (G), L Phenylalanine-^{14}C (G), L Proline-^{14}C (G), L Serine-3-^{14}C, L Serine-^{14}C (G), L Threonine-^{14}C (G), DL Tryptophan (Indolyl-Alanine-3-^{14}C), L Tyrosine-^{14}C (G), DL Valine-1-^{14}C, DL Cystine-^{35}S and DL Methionine-^{35}S After a further period of 19 hours the rats were sacrificed and their livers removed and homogenized in 2 ml of water

Preparation of polar dyes, chromatography and counting techniques

After adding an aliquot of 0 5 M pH 4 0 acetate buffer the liver proteins were precipitated by boiling for three minutes   The precipitated proteins were collected on hardened filter paper, washed with ethanol and then extracted for 48 hours with ethanol in a Soxhlet apparatus   The extracted powders were then digested in ethanolic KOH at 80° C for 20 hours and the digests extracted twice with 1   5 ethanol-ethyl ether by the method of Miller and Miller (1947)   The combined ethereal extracts were transferred to small beakers   KOH dissolved in the ethereal extracts was precipitated as carbonate by the addition of a small piece of dry ice and the extract was evaporated to dryness, initially over a water bath and finally *in vacuo*   The residues (apart from potassium carbonate) were transferred by dissolving in 1   5 ethanol-ethyl ether to 2 2 cm diameter watch glasses and any activity determined by using a Geiger Muller tube with a mica window 2 3 mg cm $^{-2}$, counting was performed at infinite thinness for a minimum time of 30 minutes for each sample (background 7–10 counts per minute)

The samples were transferred to Whatman No 1 paper with a small amount of ethanol   The total amount of polar dye extracted from one liver was introduced on to the paper in three spots   If necessary additional unlabelled polar dye eluted from other chromatograms was added to make the spots visible   The spots were dried with a hair drier between each application and then chromatographed using the solvent system *n*-butanol, acetic acid, water 4 1 5   Prior to chromatography the spots were exposed to ammonia vapour   After chromatography and drying, the papers were exposed to HCl vapour and any areas showing a faint pink colour were cut out and eluted with ethanol in a semi-micro Soxhlet apparatus   After standing under a hood for 3–4 hours the remainder of the paper was sprayed with ninhydrin   After drying *in vacuo* the eluted material from the pink spots was then rechromatographed on Whatman No 1 paper using the system benzyl alcohol, water and once again the areas showing a faint pink colour on acidification were cut out and eluted with ethanol   All the polar dye arising from a single liver (which was run in three spots on the initial chromatograms) was then pooled and run as a single spot in the benzyl alcohol, water system   After drying, the diluted material was transferred to watch glasses and counted as before at infinite thinness, for a minimum time of 30 minutes   The density of material on the sources counted was not greater than 10 μg cm $^{-2}$ The material counted in each case was

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* Obtained from the Radiochemical Centre, Amersham, Bucks , England

thus the total amount of polar dye derived from a single liver   Counting was performed for at least half an hour with each source , background varied between 8 and 10 counts per minute   The amount of azo dye present in each source was estimated spectrophotometrically in HCl-ethanol at 520 mμ A solution of 3′MeDAB in acid-ethanol was used as a standard

In all cases control spots of known unlabelled amino acid were run on the same piece of paper with the dye-labelled amino acid samples   As a result of a preliminary screening experiment tryptophan, valine, histidine and proline were subjected to further study   Using these amino acids, methionine and cystine, litter mates were given either a labelled amino acid alone or a labelled amino acid plus carcinogen and their livers subsequently hydrolyzed and extracted in an identical manner   Both samples were then chromatographed on the same sheet of paper and corresponding areas were cut out, eluted and counted

All chromatography was performed at 23 ± 2° C

RESULTS

The polar derivatives of 3′MeDAB prepared from livers of rats given ^{14}C labelled alanine arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine and tyrosine showed little or no activity   All of these experiments were run at least in duplicate , in some cases the procedure was carried out four times

The polar dyes obtained from rats given labelled tryptophan, valine, methionine and cystine showed appreciable activity (Table I)   While material prepared

TABLE I —*Showing Activities of Polar Dyes Obtained from Rats given Labelled Tryptophan, Valine, Cystine and Methionine*

Amino acid	Number of rats	Mean counts per minute per mg DF polar dye	Level of statistical significance of difference between group given carcinogen and control group (*t* test)
Tryptophan	8	4495±285*	<1%
Valine	7	3268±252*	<1%
Cystine	4	2887±356*	6%
Methionine	4	4420±962	Not significant

* = Standard deviation

in the same way from rats given labelled tryptophan only (without 3′MeDAB) or labelled valine only showed very low activity, that prepared from rats given ^{35}S labelled methionine or cystine only showed some activity

Rf values of amino acids and aminoazo dyes are shown in Table II

DISCUSSION

Interpretations drawn from studies of this type must be viewed within the limitations of the technique   The metabolic interconversion of amino acids is well established so that if the polar derivative of 3′MeDAB prepared from rats given a particular ^{14}C labelled amino acid shows a significant but low activity, this should

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TABLE II —*Showing Rf Values of Amino Acids and Azo Dyes in Solvent Systems Used*

Substance	Benzyl alcohol-water	n-Butanol, acetic acid water—4 1 5
Alanine	0 04	0 39
Arginine	0 01	0 19
Aspartic acid	0 00	0 33
Glutamic acid	0 00	0 37
Glycine	0 02	0 33
Histidine	0 02	0 19
Iso Leucine	0 18	0 70
Leucine	0 20	0 72
Lysine	0 00	0 18
Phenylalanine	0 37	0 66
Proline	0 12	0 43
Serine	0 01	0 31
Threonine	0 02	—
Tryptophan	0 29	0 61
Tyrosine	0 14	0 53
Valine	0 11	0 56
Cystine	0 00	—
Methionine	0 17	0 57
Polar dye*	0 80	0 75
3'MeDAB	0 94	0 94
3'MeMAB	1 00	0 93
3'MeAB†	1 00	0 94

* The dye recovered after hydrolysis of the liver proteins has polar properties as demonstrated by Miller and Miller (1947) and was called by them polar dye Its exact structure has not been determined as yet
† Presented by Dr J A Miller

be considered as being due to the amino acid given being converted to another amino acid which becomes bound On the other hand if the polar dye obtained, after giving a particular labelled amino acid, shows high activity this might possibly indicate that this amino acid marks the site of attachment of dye to protein In order to reduce this interconversion of amino acids the time taken from administration of the dye and labelled amino acids should be reduced to a minimum The observation of Gelboin, Miller and Miller (1958) that the polar dye formed within hours of giving a large dose of 3'MeDAB is similar to that formed on prolonged feeding of lower levels of dye, offers an opportunity of greatly reducing this time but still retaining a high level of bound dye Thus the time of 19 hours was determined by the interval required to achieve sufficient dye binding to enable recognition of the dye on the chromatograms This is considerably longer than the time required for maximal labelling of proteins by giving amino acids

Infant rats were used in order to get the maximum concentration of labelling of polar dye with associated economy of isotopes Since Gelboin, Miller and Miller have correlated the rapid achievement of a high level of bound dye with the rate of protein synthesis the rats used were fasted but returned to the mother shortly before injection

Preliminary investigations were made concerning the toxicity of large amounts of certain unlabelled amino acids to infant rats in an endeavour to investigate the possibility of decreasing the conversion of one amino acid into another in subsequent experiments using isotopes , for example, in view of the conversion

of serine to cystine it was considered that the administration of a large excess of unlabelled cystine with labelled serine would decrease the formation of labelled cystine from the serine   However, as the combined injections of 3'MeDAB and a large excess of an amino acid frequently was found to be toxic to suckling rats, this procedure was not adopted in the experiments using isotopically labelled amino acids

In all experiments employing isotopic techniques most stringent purification methods are essential   The extracted dyes were purified by chromatography using the systems n-butanol, acetic acid, water 4 1 5 and benzyl alcohol-water   Control spots of free amino acids were run on the same sheets of paper and these showed a clear separation between the polar dye and the free amino acids   The Rf values of the amino acids and aminoazo dyes are sufficiently different, in the solvent system used, to obtain a good separation (Table II)   In each case a ninhydrin reaction carried out after cutting out the polar dye spots showed no ninhydrin positive material at the margin of the spot

Before attributing activity to the presence of any amino acid as a substituent in the polar dye molecule it is necessary to demonstrate that the material, prepared in the same way from the livers of rats given labelled amino acid but no carcinogen, shows little or no activity   This excludes the possibility that the activity observed is due to any small peptide or other metabolite of the amino acid in question with Rf values similar to that of the polar bound dye

Appreciable activity was obtained only with the polar dyes derived from rats given labelled tryptophan, valine, cystine or methionine   In the cases of cystine and methionine, however, some activity was also found in corresponding material prepared from rats given labelled amino acid only

Application of the t-test to the results (group given 3'MeDAB plus labelled amino acid and group given labelled amino acid alone) gave values which were significant at the 1 per cent level for tryptophan and valine and at the 6 per cent level for cystine   No statistically significant difference between the methionine groups could be detected

The results obtained suggest that tryptophan, valine and possibly cystine (or cysteine) residues are associated with the polar bound dye

Since in these experiments no activity was found in the polar dye recovered from rats given dye and either labelled tyrosine or its precursor phenylalanine, it is difficult to reconcile these results with the suggestion of Kusama and Terayama (1957), Terayama and Kusama (1957), Kusama et al (1958) and Terayama et al (1958), that a tyrosine residue is involved

It is appreciated that these results are based on experiments on few animals   Further work is contemplated

SUMMARY

1   Suckling rats have been given 3'MeDAB and individual labelled amino acids   The recovered polar dyes have been purified by chromatography and examined for activity

2   Activity has been found in the polar dyes prepared from rats given labelled tryptophan, valine, cystine and methionine

3   It is suggested that tryptophan, valine and possibly cystine (cysteine) residues are the substituents in the polar derivatives

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INDIRECT EFFECT OF X-RADIATION ON THE RESPIRATORY METABOLISM OF EHRLICH ASCITES TUMOUR CELLS AND MITOCHONDRIA

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It has been found previously (Silk, Hawtrey and Macintosh, 1958) that Ehrlich ascites cells show lower oxygen uptake and aerobic glycolysis in a medium of peripheral lymph drained from X-irradiated dog tissues than in a medium of normal peripheral dog lymph Since the ascites cells themselves were not irradiated, an " indirect " action of radiation was postulated to account for the observed effects on respiratory metabolism which operate through the environmental medium

In a further examination of this " indirect " action of radiation, the respiratory metabolism of intact Ehrlich ascites cells and of Ehrlich ascites cell mitochondria has been compared in media previously incubated with mouse antero-lateral abdominal wall and in the same media given 1000 roentgen X-irradiation in contact with the tissue before incubation Although the glycolysis of intact cells is little different in the non-irradiated and X-irradiated media, the oxygen uptake of both intact cells and mitochondria is markedly influenced Measurements of respiration have been made in both bicarbonate and phosphate buffered media As in previous work (Silk, Hawtrey and Macintosh, 1958) the effects are found to be opposite in the two buffer systems

The reasons for this " indirect " effect of radiation has been sought in the chemical composition of the environmental media before and after radiation It would appear that factors liberated by the irradiated mouse antero-lateral abdominal wall are capable of influencing the respiration rate of Ehrlich ascites cells and mitochondria

MATERIALS AND METHODS

Mouse antero-lateral abdominal wall

Mice were killed by cervical fracture A median incision was then made in the ventral aspect of the abdomen Flaps of skin and subcutaneous tissue were dissected away up to the dorsal aspect of the abdominal cavity The remainder of the antero-lateral abdominal wall consisting of peritoneum, subserous tissue and fascia with layers of muscle and overlying connective tissue, was then excised for experiment The tissue was cut into portions approximately 1 cm square,

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and stored in ice cold normal saline +0 2 per cent w/v glucose until ready for incubation with various media

Preparation of media for incubation with mouse antero-lateral abdominal wall

(a) *Ehrlich ascites plasma* —Citrated (5 mg /ml) Ehrlich ascites fluid was centrifuged at 4° C , to give clear ascites plasma free of cells   The bicarbonate content of an aliquot was determined by acidification in Warburg manometers filled with 95 per cent O_2 + 5 per cent CO_2, and the calculated amount of 0 75 M $NaHCO_3$ solution was then added to the main bulk to give a final bicarbonate concentration of approximately 0 017 M   The pH values of all samples used lay within the range pH 7 2–7 4 when measured in contact with a gas mixture containing 95 per cent O_2 + 5 per cent CO_2, and agreed generally to within 0 2 of a pH unit for corresponding batches of non-irradiated and 1000 roentgen X-irradiated media   For use as a medium in which to determine both the glycolysis and respiration of intact Ehrlich ascites cells, the plasma was fortified with 0 25 per cent w/v glucose before incubation with mouse tissue   For use as a medium in which to determine only the O_2 uptake of intact cells, 1 30 ml of 0 3 M potassium succinate was added to each 60 ml in place of the glucose   This amount of succinate was adequate to serve as substrate for the mouse antero-lateral abdominal wall tissue during incubation, and also for respiration of the Ehrlich cells allowed to metabolise in the medium after incubation

(b) *Ringer bicarbonate-succinate* —The medium contained 1 30 ml of 0 3 M potassium succinate per 60 ml of standard Ringer bicarbonate solution

(c) *Ringer phosphate-succinate* —The medium contained 1 30 ml of 0 3 M potassium succinate per 60 ml of standard Ringer phosphate solution

(d) *Phosphate buffered medium* —The following medium was prepared   phosphate buffer (pH 7 4), 0 067 M , $MgCl_2$, 0 009 M , KCl, 0 015 M , glucose, 0 015 M   sucrose, 0 044 M

Radiation and incubation with mouse antero-lateral abdominal wall

Precautions were taken to ensure sterility during all operations

Aliquots (30 ml) of the required medium (a), (b), (c) or (d), were placed in pairs of 250 ml conical flasks   Antero-lateral abdominal wall sections from 6 mice (approximately 2 4 g wet wt) were placed in each of the flasks   One flask of each pair was then given 1000 roentgen X-irradiation (220 kV, 15 mA, filtered through 0 2 mm Sn + 0 25 mm Cu + 1 0 mm Al) at the rate of 160 roentgen/min delivered through the base of the vessel

Both the irradiated and non-irradiated flasks of each pair were then incubated 3 hours with slow shaking on a Warburg bath at 37° C   During incubation of bicarbonate media 200 c c /min of water-saturated 95 per cent O_2 + 5 per cent CO_2 gas mixture was blown through the flasks   Suspensions in phosphate buffered medium were similarly gassed with 200 c c /min pure O_2

After incubation the media were freed of all cells by centrifugation at 2500 revs/min for 15–20 minutes

Ascites plasma (a) —The bicarbonate concentration was re-adjusted to 0 017 M as described above   For use as a medium in which to determine glycolysis, extra glucose was added in amount equivalent to the bicarbonate lost through fermentative lactic acid production during incubation with the tissue

Ringer bicarbonate-succinate (b) —The bicarbonate concentration was adjusted to 0 195 M after incubation No extra succinate was added

Ringer phosphate-succinate (c) *and Phosphate buffered medium* (d) were carefully triturated to pH 7 5 with KOH after incubation and were used without further addition of succinate or glucose

All media were filtered through sintered glass (Porosity 5) and were stored in the cold before use

Deproteinisation of Ehrlich ascites plasma after incubation with mouse antero-lateral abdominal wall

Immediately after incubation the plasma was deproteinised by addition of 4 vols cold acid-free ethanol After standing overnight at 4° C the precipitate was removed by centrifugation Alcohol was removed from the supernatant by concentration under reduced temperature and pressure to $\frac{1}{4}$ of the original plasma volume The solution was then made up to the original plasma volume by addition of distilled water The bicarbonate concentration was re-adjusted to 0 017 M as above, and the medium passed through a sintered glass (Porosity 5) filter before use

Ehrlich mouse ascites carcinoma cells

The tumour was kindly supplied by Dr Kanematsu Sugiura of the Sloan-Kettering Institute, N Y , and has been maintained in Swiss albino mice which were used between the 9th and 12th day after inoculation

The total cells present in a chilled 10 ml sample of citrated (5 mg /ml) ascites fluid was removed by centrifugation at 4° C For experiments with bicarbonate buffered media the cells were washed by re-suspending in 2–3 vols Ringer bicarbonate-succinate solution (b) For experiments with phosphate buffered media the cells were washed in the appropriate solution (c) or (d) Weighed portions of each washed cell mass were then thoroughly slurried with aliquots of corresponding non-irradiated and X-irradiated media (150 mg /4 5 ml for bicarbonate buffered media or 420 mg /7 0 ml for phosphate buffered media) Aliquots (2 0 ml) of the resulting suspensions were used for manometric measurement of $Q_G^{O_2}$ and Q_{O_2}

The following abbreviations are used in the text

$Q_G^{O_2}$	= Aerobic glycolysis in terms of μl CO_2 per mg dry weight of tissue per hour at 37° C
Q_{O_2} (intact cells)	= μl O_2 uptake per mg dry weight of cells per hour at 37° C
Q_{O_2} (mitochondria)	= μl O_2 uptake per mg protein per hour at 30° C
ADP	= adenosine diphosphate
AMP	= adenosine monophosphate
DPN	= diphosphopyridine nucleotide
EDTA	= ethylene diamine tetra acetic acid
TCA	= trichloro acetic acid

Bicarbonate containing media were equilibrated with 95 per cent O_2 + 5 per cent CO_2 in a small pressure bomb at 30 p s i before use Phosphate buffered media were equilibrated with pure O_2

Ehrlich ascites cell mitochondria

These particulates were isolated in 0 25 M sucrose according to the method of Hawtrey and Silk (1960) Saline washed Ehrlich ascites cells were previously

swollen 15 minutes in hypotonic Ringer phosphate solution and then lysed into pure water using a Dounce homogeniser for 3 0 minutes   Immediately after 3 minutes, concentrated sucrose solution was added to give a 0 25 M sucrose homogenate from which the mitochondria were isolated by differential centrifugation in the customary manner   Twice washed mitochondria free of " fluffy layer " were used for all experiments   Each 1 0 ml of the final suspension in 0 25 M sucrose contained 3–11 mg of mitochondrial protein determined according to Cleland and Slater (1953)

Aerobic glycolysis and oxygen uptake of intact Ehrlich ascites cells in bicarbonate buffered media

Aerobic glycolysis and oxygen uptake were determined simultaneously by the method of Dixon and Keilin (1933) using Barcroft differential manometers   The precautions described by Elliott and Schroeder (1934) and Dixon (1951) were observed   Measurements were made at 37° C and the flasks were gassed with water-saturated 95 per cent O_2 + 5 per cent CO_2 during a 15 minute equilibration period   The side arms contained 0 5 ml of 1 0 M HCl and the stopper cavities were filled with 40 per cent KOH   Clerici solution (P_0 = 2500) was used in the manometers

The two flasks of each manometer contained identical aliquots of either (a) the Ehrlich ascites cell suspension in medium incubated with tissue but not irradiated, or (b) the Ehrlich ascites cell suspension in medium incubated with mouse abdominal wall tissue after 1000 roentgen X-irradiation

Experiments were conducted over periods of 40–50 minutes during which time the rates of glycolysis and respiration remained apparently undiminished

The pH at the beginning and end of each experiment was calculated using the Henderson-Hassalbalch equation with the values $pK^1 = 6\ 1$, $\alpha = 0\ 51$ (c c CO_2 dissolved per ml of medium at 37° C and 760 mm) for ascites serum and $pK^1 = 6\ 32$, $\alpha = 0\ 57$ for non-proteinaceous media   The initial pH values of all suspensions were found to lie within the range pH 7 2–7 4

Retention curves necessary to correct the observed glycolysis for the amount of CO_2 retained by the medium and not registered on the manometer were determined by the method of Brekke and Dixon (1937)   The values of R/Δ pH for the X-irradiated medium were on an average 14 per cent lower than for the corresponding non-irradiated medium

Oxygen uptake and P/O ratio of Ehrlich ascites cell mitochondria in phosphate buffered media incubated with mouse antero-lateral abdominal wall

Corresponding series of Warburg flasks were set up with (a) 2 0 ml aliquots of phosphate buffered medium incubated with mouse tissue but not irradiated, and (b) 2 0 ml aliquots of corresponding medium incubated with tissue after 1000 roentgen X-irradiation   To each flask was added 0 26 ml of the following solution KF, 0 173 M , $MgCl_2$, 0 023 M , ADP, 0 029 M , AMP, 0 012 M , EDTA, 0 023 M , cytochrome-c, 1 13 × 10^{-4} M , DPN, 0 0028 M   The side arms of each flask contained 0 24 ml of 0 3 M glucose plus 1–2 mg of crystalline hexokinase, and the centre wells 0 2 ml of 40 per cent KOH solution with a roll of filter paper

After cooling the flasks in ice, 0 4 ml of the mitochondrial suspension in 0 25 M sucrose was added to each vessel

Final concentrations in each flask were as follows potassium phosphate buffer (pH 7 4), 0 045 M , KF, 0 015 M , MgCl₂, 0 008 M , ADP, 0 0025 M , AMP, 0 001 M , EDTA, 0 002 M , glucose, 0 034 M , KCl, 0 01 M , sucrose 0 062 M , DPN, 2 4 × 10⁻⁴ M , cytochrome-c, 9 8 × 10⁻⁶ M , hexokinase, 1–2 mg (plus unknown amounts of lactic acid, amino acids, substrates, etc , resulting from incubation of part of the flask contents with mouse antero-lateral abdominal wall)

Experiments were conducted at 30° C over periods of 25–35 minutes with air as the gas phase An equilibration time of 13 minutes on the manometer bath was allowed before closing the taps and adding the side-arm contents The reaction was stopped by addition of 1 0 ml of 30 per cent TCA to each flask which was then placed in ice Phosphate uptake was determined by the method of Fiske and Subbarow (1925) on the cold TCA filtrate

Amino acid analysis

Samples of corresponding non-irradiated and X-irradiated media were deproteinised by addition of 4 vols ethanol After standing overnight at 4° C and centrifuging, 0 2 ml aliquots of the supernatant were analysed for total free amino acid and peptide content by the quantitative ninhydrin method of Troll and Cannan (1953) using a standard leucine curve

Protein analysis

Duplicate aliquots of corresponding non-irradiated and X-irradiated media were analysed for total protein content according to the biuret method of Gornall, Bardawill and David (1949)

Assay of materials

Hexokinase was used in the crystalline form supplied by General Biochemicals Inc , Chagrin Falls, Ohio, and was assayed (a) by hexose disappearance according to Somogyi (1952) and (b) by measurement of acid labile phosphorus disappearance according to Crane and Sols (1955) Preparations were found to have an activity of approximately 150 units per mg

Cytochrome-c was used as a 1 per cent solution in isotonic saline as supplied by General Biochemicals Inc and was found to contain 5 87 × 10⁻⁷ moles per ml when assayed according to Umbreit, Burris and Stauffer (1957)

AMP and *ADP* were used as supplied by General Biochemicals Inc and by the H M Chemical Co of Los Angeles, California, respectively

DPN was obtained from General Biochemicals Inc and was assayed 85 per cent pure by a modification of the dithionite reduction method of Brodie (1955) using 0 2 M phosphate buffer instead of 0 1 M

All other substrates and chemicals were of Analytical Reagent grade, and where necessary were neutralised to pH 7 4 with potassium hydroxide before use

RESULTS

(1) *Aerobic glycolysis of Ehrlich ascites cells in ascites plasma incubated with mouse antero-lateral abdominal wall*

The aerobic glycolysis of Ehrlich ascites cells was compared in 6 corresponding batches of ascites plasma incubated with abdominal wall tissue from healthy mice (a) non-irradiated and (b) 1000 roentgen X-irradiated before incubation

The results in Table I show that the glycolysis was on an average 15 per cent lower in the X-irradiated medium

TABLE I —*Aerobic Glycolysis of Ehrlich Ascites Cells in Ascites Plasma Incubated with Tissue, (a) Not Irradiated and* (b) 1000 *Roentgen X-Irradiated before Incubation*

| Batch No | Glycolysis $Q_G^{O_2}$ | | Percentage difference |
	Not irradiated	X irradiated	
1	39 5	36 6	−7 3
	30 2	31 6	+4 6
2	36 6	27 9	−23 8
	41 4	20 2	−51 2
3	23 0	21 5	−5 8
	24 8	22 2	−10 5
4	31 1	24 8	−12 7
	28 6	24 3	−15 0
5	31 8	30 3	−4 7
	38 6	27 5	−28 7
6	31 8	26 1	−17 9
	31 0	27 6	−11 0
Average	32 4±4 4*	26 7±3 5	−(15 3±10 1)

* Average deviation from mean

(2) *Oxygen uptake of Ehrlich ascites cells in ascites plasma incubated with mouse antero-lateral abdominal wall*

The oxygen uptake of Ehrlich ascites cells was compared in 5 corresponding batches of ascites plasma incubated with abdominal wall tissue from healthy mice (a) non-irradiated and (b) 1000 roentgen X-irradiated before incubation

The results in Table II show that the oxygen uptake was on an average 43 per cent higher in the media X-irradiated with tissue before incubation

TABLE II —*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Incubated with Tissue* (a) *Not Irradiated and* (b) 1000 *Roentogen X-irradiated before Incubation*

| Batch No | Oxygen uptake Q_{O_2} | | Percentage difference |
	Not irradiated	X irradiated	
1	2 01	4 55	+126 3
	3 03	6 04	+99 3
2	6 57	7 26	+10 5
	4 78	5 77	+20 7
3	6 07	8 10	+33 4
	5 38	7 53	+40 0
4	5 54	6 68	+20 6
	6 70	8 92	+33 1
5	5 84	7 32	+25 4
	6 25	7 63	+22 1
Average	5 22±1 17*	6 98±0 98	+(43 1±27 8)

* Average deviation from mean

(3) *Aerobic glycolysis of Ehrlich ascites cells in ascites plasma incubated with antero-lateral abdominal wall from tumour-bearing mice*

For these experiments the antero-lateral abdominal wall was removed from mice bearing a 10-day Ehrlich ascites tumour Any small areas of solid tumour formation were carefully removed and macroscopically the tissue appeared normal Incubation with ascites plasma was carried out as described for antero-lateral abdominal wall from healthy mice

The aerobic glycolysis of Ehrlich ascites cells was then compared in 3 batches of ascites plasma incubated with the tissue (a) non-irradiated and (b) 1000 roentgen X-irradiated before incubation

The results in Table III show that the glycolysis was on an average very slightly lower in the X-irradiated medium than in the corresponding non-irradiated control

TABLE III —*Aerobic Glycolysis of Ehrlich Ascites Cells in Ascites Plasma Incubated with Antero-lateral Abdominal Wall from Tumour-bearing Mice* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

| Batch No | Glycolysis $Q_G^{O_2}$ | | Percentage difference |
	Not irradiated	X irradiated	
1	33 2	29 2	−12 0
	38 1	37 1	−2 6
2	32 8	33 5	+2 1
3	35 2	33 8	−4 0
	35 0	35 2	+0 6
Average	34 9±1 5*	33 8±1 9	−(3 3±2 8)

* Average deviation from mean

(4) *Oxygen uptake of Ehrlich ascites cells in ascites plasma incubated with antero-lateral abdominal wall from tumour-bearing mice*

Antero-lateral abdominal wall was removed from mice bearing a 10-day Ehrlich ascites tumour, and was incubated with ascites plasma as above

The oxygen uptake of Ehrlich ascites cells was then compared in 2 batches of ascites plasma incubated with the tissue (a) non-irradiated and (b) 1000 roentgen X-irradiated before incubation

The results in Table IV show that the oxygen uptake was on an average 50 per cent higher in the medium incubated with tissue after X-irradiation The magnitude of the effect decreased with storage time of the media before use, indicating lability of the factor(s) responsible

(5) *Oxygen uptake of Ehrlich ascites cells in ascites plasma deproteinised after incubation with mouse antero-lateral abdominal wall*

Ehrlich ascites plasma fortified with potassium succinate was incubated with healthy mouse antero-lateral abdominal wall (a) without radiation and (b) with 1000 roentgen X-irradiation before incubation The media were subsequently deproteinised

The oxygen uptake of Ehrlich ascites cells was then compared in 4 corresponding batches of medium prepared with and without radiation

TABLE IV —*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Incubated with Antero-lateral Abdominal Wall from Tumour-bearing Mice* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated Before Incubation*

	Oxygen uptake Q_{O_2}			
Batch No	Not irradiated	X-irradiated	Percentage difference	Storage time† after incubation (days)
1	3 96	5 58	+41 0	5
2	3 00	7 80	+160 0	3
	4 72	4 91	+4 0	8
	5 39	6 21	+17 0	10
Average	4 27±0 79*	6 13±0 88	+(50 5±9 5)	—

* Average deviation from mean
† Media were stored at −30° C before experiment

The results in Table V show that the oxygen uptake was on an average 32 per cent higher in the medium incubated with tissue after X-irradiation

TABLE V —*Oxygen uptake of Ehrlich Ascites Cells in Ascites Plasma Deproteinised After Incubation with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

	Oxygen uptake Q_{O_2}		
Batch No	Not irradiated	X irradiated	Percentage difference
1	9 00	10 93	+21 5
2	4 15	5 78	+39 2
	5 15	6 85	+33 0
3	7 05	11 10	+57 5
	7 65	9 90	+29 6
4	9 83	11 82	+20 4
	8 86	10 64	+20 2
Average	7 38±1 66*	9 57±1 86	+(31 6±9 9)

* Average deviation from mean

(6) *Oxygen uptake of Ehrlich ascites cells in Ringer bicarbonate solution incubated with mouse antero-lateral abdominal wall*

In these experiments Ringer bicarbonate fortified with potassium succinate (solution (b)) was incubated with healthy mouse antero-lateral abdominal wall (a) without radiation and (b) with 1000 roentgen X-irradiation before incubation

The oxygen uptake of Ehrlich ascites cells was then compared in 4 corresponding batches of medium prepared with and without radiation

The results in Table VI show that the oxygen uptake was on an average 14 per cent higher in the medium incubated with tissue after X-irradiation

(7) *Oxygen uptake of Ehrlich ascites cells in ascites plasma deproteinised after 3 hours' incubation at 37° C without tissue*

Batches of succinate fortified Ehrlich ascites plasma were divided into two portions, one of which was given 1000 roentgen X-irradiation Both samples were

TABLE VI —*Oxygen Uptake of Ehrlich Ascites Cells in Ringer Bicarbonate Solution Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

	Oxygen uptake Q_{O_2}		
Batch No	Not irradiated	X-irradiated	Percentage difference
1	11 89	12 41	+4 4
2	6 92	8 72	+26 1
3	9 33	10 62	+13 8
	9 16	10 02	+9 4
4	6 96	7 90	+13 5
	7 02	8 34	+18 8
Average	8 55±1 58*	9 67±1 35	+(14 3±5 4)

* Average deviation from mean

incubated 3 hours at 37° C under O_2/CO_2 gas mixture and subsequently deproteinised

The oxygen uptake of Ehrlich ascites cells was compared in 4 corresponding batches of the deproteinised fluid prepared with and without radiation

The results in Table VII show that the oxygen uptake was almost identical in corresponding non-irradiated and X-irradiated media

TABLE VII —*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Deproteinised after* 3 *hours' Incubation at* 37° C *Without Tissue* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

	Oxygen uptake Q_{O_2}		
Batch No	Not irradiated	X-irradiated	Percentage difference
1	9 10	9 72	+6 8
2	8 65	8 72	+0 8
	11 20	11 05	—1 3
3	9 78	9 64	—1 4
	8 95	8 50	—5 0
4	9 30	9 10	—2 1
	7 10	7 05	—0 7
Average	9 15±0 80*	9 11±0 88	—(0 4±2 2)

* Average deviation from mean

(8) *Oxygen uptake of Ehrlich ascites cells in phosphate buffered media incubated with mouse antero-lateral abdominal wall*

Three distinct series of experiments were carried out using phosphate buffered media

(1) Phosphate buffered medium containing excess glucose (solution (d)) was incubated with healthy mouse antero-lateral abdominal wall (a) without radiation and (b) with 1000 roentgen X-irradiation before incubation The oxygen uptake of Ehrlich ascites cells was then compared in the media prepared with and without radiation

(2) As above, except that 0 5 ml of 0 3 M potassium succinate was added to each 70 ml of phosphate buffered medium (d) either before or after incubation with mouse tissue

(3) As above, except that Ringer phosphate-succinate medium (c), 1 e containing no glucose, was used for incubation with the mouse tissue

Oxygen uptake of ascites cells was measured in triplicate in each batch of corresponding non-irradiated and X-irradiated media   Experiments were conducted at 37° C over periods of 40 minutes, during which time respiration remained undiminished   The gas phase was pure oxygen

Table VIII shows that in phosphate buffer with glucose as substrate, the oxygen uptake of ascites cells was on an average 14 7 per cent lower in the medium incubated with tissue after X-irradiation   In phosphate buffer containing glucose and succinate as substrates, the effect was less pronounced with 4 4 per cent average lowering of oxygen uptake in the X-irradiated medium   Only 1 per cent average lowering was observed in phosphate medium with no glucose but succinate added prior to incubation

TABLE VIII —*Oxygen Uptake of Ehrlich Ascites Cells in Phosphate Buffered Media Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Medium and substrate	No of corresponding batches	No of experi ments	Oxygen uptake Q_{O_2}		Percentage difference
			Not irradiated	X irradiated	
Phosphate buffered medium (d) (glucose)	2	6	4 71±0 31	4 02±0 23*	—(14 7±0 8)
Phosphate buffered medium (d) (glucose + succinate)	3	9	5 62±0 31	5 38±0 27	—(4 4±0 3)
Ringer phosphate me dium (c) (succinate)	3	9	10 08±0 42	9 99±0 40	—(1 0±0 8)

* Average deviation from mean

(9) *Oxygen uptake of Ehrlich ascites cells in Ringer phosphate solution incubated for various times with mouse antero-lateral abdominal wall*

The Q_{O_2} of Ehrlich ascites cells was measured in succinate-fortified Ringer phosphate solution (c) which had been incubated with healthy mouse antero-lateral abdominal wall for varying times

The results in Table IX show that, within the limits of experimental accuracy, the Q_{O_2} values of ascites cells were identical in media incubated between 1–5 hours with mouse tissue

(10) *Oxygen uptake of Ehrlich ascites cell mitochondria in phosphate buffered medium incubated with mouse antero-lateral abdominal wall*

The oxygen uptake of Ehrlich ascites cell mitochondria was compared in 7 corresponding batches of phosphate buffered medium incubated with healthy mouse tissue (a) without radiation and (b) with 1000 roentgen X-irradiation before incubation   Media were triturated to pH 7 5 with KOH after incubation

Mitochondria showed a negligible endogenous oxygen uptake in phosphate buffered medium which had not been incubated with mouse antero-lateral abdominal wall and to which no citric acid cycle substrates had been added   However

TABLE IX —*Oxygen Uptake of Ehrlich Ascites Cells in Ringer Phosphate Solution Incubated for 1–8 hours with Mouse Antero-lateral Abdominal Wall   No Irradiation*

Time of incubation (hours)	Oxygen uptake Q_{O_2}	Average Q_{O_2}	Percentage increase above control
0 (Control)	7 1 / 7 4	} 7 3	—
1	7 7 / 8 1	} 7 9	8 2
2	7 9	7 9	8 2
3	7 6 / 8 2	} 7 9	8 2
5	7 9 / 7 8	} 7 9	8 2
8	8 2 / 8 8	} 8 5	16 5

the mitochondria showed considerable oxygen uptake in the same medium after incubation with mouse tissue   This was apparently due to liberation of oxidisable substrates, from the tissue into the medium during incubation

The results in Table X show that the oxygen uptake of Ehrlich ascites mitochondria was on an average 20 per cent lower in the medium incubated with tissue after irradiation, than in the control incubation medium prepared without irradiation

As an additional part of each experiment the biochemical intactness of the mitochondria was checked by measuring their oxygen uptake in phosphate buffered medium fortified with 0 01 M potassium succinate as substrate (cf Hawtrey and Silk, 1960)   Batches showing Q_{O_2} values in the range 85–130 were used for experiment

TABLE X —*Oxygen Uptake of Ehrlich Ascites Cell Mitochondria in Phosphate Buffered Medium Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No	Oxygen uptake Q_{O_2} Not irradiated	X irradiated	Percentage difference
1	62 7 / —	52 8 / 56 4	} −12 9
2	100 1 / 93 0	81 0 / 79 3	} −17 0
3	38 7 / 38 6	30 8 / 32 8	} −17 7
4	41 9 / 47 5	34 1 / 32 7	} −25 3
5	70 5 / 96 2	56 8 / 58 4	} −30 9
6	94 9 / 77 6	80 5 / 71 7	} −11 0
7	76 1 / 72 0	58 2 / 47 0	} −29 0
Average	70 0±18 5*	55 2±14 4	−(20 5±6 7)

* Average deviation from mean

(11) *P/O ratio of Ehrlich Ascites cell mitochondria in phosphate buffered medium incubated with mouse antero-lateral abdominal wall*

As part of the experiments to measure O_2 uptake of Ehrlich ascites cell mitochondria, the *P/O* ratios of these particulates were determined in 7 corresponding batches of phosphate buffered medium incubated with healthy mouse tissue (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation

The results in Table XI show that the *P/O* ratio was on an average 16 per cent lower in the medium incubated with tissue after X-irradiation　Since no citric acid cycle metabolites were added at any stage in the experiments, the *P/O* ratios shown are those of the substrate(s) liberated from the mouse tissue into the phosphate buffered medium during incubation

TABLE XI —*P/O Ratio of Ehrlich Ascites Cell Mitochondria in Phosphate Buffered Medium Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

| | P/O ratio | | Percentage difference |
Batch No	Not irradiated	X irradiated	
1	1 16 —	1 11 0 97	} −10 4
2	1 13 1 14	1 09 1 01	} −8 1
3	1 14 1 11	1 10 0 97	} −8 0
4	1 24 1 08	0 84 0 87	} −26 7
5	1 29 1 15	0 94 1 01	} −20 5
6	0 91 —	0 84 0 77	} −12 0
7	0 98 1 14	0 85 0 71	} −26 4
Average	1 12±0 07*	0 93±0 10	−(16 0±7 3)

* Average deviation from mean

(12) *Effect of radiation on the protein and amino acid analysis of media after incubation with mouse antero-lateral abdominal wall*

Table XII shows the total protein content, and the content of free amino acids and peptides present in corresponding batches of various media incubated with mouse tissue　In all cases the incubation medium prepared after X-irradiation showed a lower protein content and a higher free amino acid and peptide content than the corresponding non-irradiated medium

DISCUSSION

Previous work (Silk, Hawtrey and Macintosh, 1958) showed that irradiation of the normal tumour-bed and stroma cells of a neoplasm, might " indirectly " affect the respiratory metabolism of non-irradiated malignant cells　The " indirect " effect was considered due to radiation induced substances from the normal cells reaching the malignant cells via the common environment of peripheral lymph

TABLE XII.—*Protein and Amino Acid Analysis of Media after Incubation with Mouse Antero-lateral Abdominal Wall (a) without Irradiation and (b) with 1000 Roentgen X-irradiation Prior to Incubation*

Medium	No of corresponding batches	Total protein (mg/ml)			Total free amino acids and peptides (mg/ml)		
		Not irradiated	X irradiated	Percentage difference	Not irradiated	X irradiated	Percentage difference
Ascites plasma incubated with mouse tissue	5	8 85±0 57*	7 83±1 23	−(15 8±12 6)	1 25±0 21	1 30±0 14	+(6 0±15 8)
Ascites plasma deproteinised after incubation with mouse tissue	4	—	—	—	0 88±0 37	0 96±0 14	+(48 7±60 4)
Ascites plasma deproteinised after incubation without tissue	3	—	—	—	0 08±0 16	1 04±0 17	+(26 0±7 7)
Phosphate buffered medium (d) incubated with mouse tissue	8	1 02±0 18	0 85±0 21	−(16 6±7 2)	0 45±0 13	0 58±0 17	+(28 6±6 7)

* Average deviation from mean

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For the present investigation, mouse antero-lateral abdominal wall tissue has been selected to represent part of the normal tumour-bed and stroma of the Enrlich ascites tumour   Various physiological media have been incubated with this tissue to provide the environmental fluids through which X-irradiation has been found to influence the respiratory metabolism of non-irradiated ascites cells and mitochondria

In bicarbonate buffered media, intact ascites cells showed slightly lower aerobic glycolysis (Tables I and III) but markedly higher oxygen uptake (Tables II, IV, V and VI) in the X-irradiated medium than in the corresponding non-irradiated control   No elevation of cell respiration was observed in the case of ascites plasma deproteinised after X-irradiation and incubation in the absence of mouse tissue (Table VII)

In phosphate buffered media, the oxygen uptake of ascites cells was lower in the medium prepared with X-irradiation prior to incubation (Table VIII)   The effect is therefore opposite to that found in bicarbonate buffered media   A similar inversion of effect was observed in previous work (Silk, Hawtrey and Macintosh, 1958) and may be due to competition between respiratory and glycolytic processes for the low amount of inorganic phosphate usually available in physiological bicarbonate media (cf   Kvamme, 1958)

The respiration and P/O ratio of ascites cells mitochondria were also found to be depressed in the phosphate buffered medium incubated with mouse tissue after X-irradiation (Tables X and XI)   The effect therefore parallels that observed for intact cells in phosphate media, but is again opposite to that observed for whole cells in bicarbonate buffered media

Due to protein radiolysis, the X-irradiated incubation medium has, in each case, a lower protein content but a higher free amino acid and peptide content than the corresponding non-irradiated medium (Table XII)   This holds for X-irradiated and non-irradiated ascites plasma incubated in the absence of mouse tissue, yet the oxygen uptake of ascites cells was not different in the two media (Table VII)   Thus radiolysis of the environmental medium itself would not account for the observed effects on respiratory metabolism

The media incubated with mouse abdominal wall contain respiratory substrates liberated by the tissue   This is evidenced by the high Q_{O_2} of mitochondria in phosphate buffered media incubated without added substrates such as succinate (Table X) and by the fact that ascites cells show a higher Q_{O_2} in Ringer phosphate-succinate solution incubated with mouse tissue, than in the same medium without incubation (Table IX)   The amount of substrates liberated from the tissue during incubation times of 1–5 hours was adequate to maintain the respiration rate of ascites cells at a consistent level above the control value (Table IX)

A mere difference in quantity of liberated substrates would not however account for the difference in rates of oxygen uptake shown by ascites cells and mitochondria in corresponding batches of non-irradiated and X-irradiated media   It would therefore seem that the X-irradiated medium contains in addition some radiation induced factor(s) capable of affecting the rate of oxygen consumption   Table VIII shows that liberation of these factors may depend upon the presence of glucose in the medium incubated with mouse tissue

Bernheim, Barber, Ottolenghi and Wilbur (1958) found that certain tissues with no natural anti-oxidants form peroxides on aerobic incubation, whereas other tissues do this if the natural anti-oxidant is destroyed by radiation   Shuster

(1955) showed that methyl linoleate and linolenate hydroperoxides inhibit the oxygen uptake of Ehrlich ascites cells in Ringer phosphate solution and inhibit their glycolysis in Ringer bicarbonate solution Such peroxides also inhibit the oxidising enzymes in liver mitochondria (Ottolenghi, Bernheim and Wilbur, 1955)

Our findings concerning the radiation effect on glycolysis in bicarbonate media (Tables I and III) and on oxygen uptake in phosphate media (Tables VIII and X) are therefore compatible with the presence of peroxides in the X-irradiated medium The elevation of oxygen uptake observed in bicarbonate buffered X-irradiated medium (Tables II, IV, V and VI) does not however appear to be compatible with the known effects of peroxides on respiration The X-irradiated media were examined for organic hydroperoxides using both iodide and titanous sulphate reagents (Scholes, Weiss and Wheeler, 1956) but none were found present Spectroscopic examination of corresponding non-irradiated and X-irradiated media revealed no qualitative difference in pattern in the region 200–1800 mμ

It may thus be concluded that the environmental fluid incubated with an irradiated normal tissue representing the tumour-bed of a neoplasm, contains substances which affect the respiratory metabolism of non-irradiated malignant cells The factors responsible for this " indirect " effect are at present unknown, but do not result from radiolysis of the environmental fluid itself and must therefore originate from metabolism of the tissue irradiated in contact with this fluid

SUMMARY

1 Various proteinaceous and non-proteinaceous physiological media were incubated 3 hours at 37° C with mouse antero-lateral abdominal wall tissue (a) without radiation and (b) with 1000 roentgen X-radiation of the medium plus tissue prior to incubation

2 The repiratory and glycolytic metabolism of Ehrlich ascites cells and mitochondria was then compared in corresponding batches of media prepared with and without radiation In some experiments oxidisable substrates were added to the media, while in others substrates liberated from the mouse abdominal wall tissue served to maintain respiration of the added ascites cells or mitochondria

3 With bicarbonate buffering, intact ascites cells showed lower aerobic glycolysis and markedly elevated oxygen uptake in the X-irradiated medium

4 With phosphate buffering the opposite effect was observed Both intact ascites cells and mitochondria showed lower oxygen uptake in the X-irradiated medium Mitochondria showed lower P/O ratios in the X-irradiated medium than in the corresponding non-irradiated control

5 Since neither the cells nor the mitochondria were directly radiated, the results demonstrate an " indirect " effect of X-radiation on respiratory metabolism The effect operates via the environmental medium irradiated in contact with a tissue selected to represent the normal tumour-bed and stroma of the Ehrlich tumour

6 The " indirect " effect does not seem due to radiolysis of the environmental medium itself, nor do organic hydroperoxides appear to be responsible It is suggested that metabolic products from the irradiated tissue are liberated into the medium, and that these influence the respiration rate of added Ehrlich ascites cells and mitochondria

This work has formed part of the research being undertaken in terms of a Senior Fellowship awarded to one of us (M H S) by the National Cancer Association of South Africa

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THE DEOXYRIBONUCLEIC ACID CONTENT OF CARCINOMA OF THE UTERUS AN ASSESSMENT OF ITS POSSIBLE SIGNIFICANCE IN RELATION TO HISTOPATHOLOGY AND CLINICAL COURSE BASED ON DATA FROM 165 CASES

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RECENT technical advances in chromosome cytology have confirmed the existence of a high degree of constancy in mammalian somatic tissues (Ford, Hamerton and Mole, 1958 , Tjio and Puck, 1958) and have served to throw into sharp relief the diversity of chromosome number that has been reported in malignant tumours (Levan, 1956a , Ising and Levan, 1957 , Manna, 1957 , Klein, 1959 , Makino, Ishihara and Tonomura, 1959 , Richards and Atkin, 1960)   The association of certain congenital abnormalities in man with loss or duplication of single chromosomes (Ford, Jones, Polani, de Almeida and Briggs, 1959 , Jacobs and Strong, 1959 , Lejeune, Gautier and Turpin, 1959) further demonstrates that profound developmental disturbances may result from such changes   This could be inferred from studies on lower animals and plants, but is only now susceptible of direct investigation in man   On the other hand, though some mammalian tumours have an apparently normal chromosome complement (Klein, 1959), the great majority of those for which reliable counts are available present differences in chromosome number from the normal, and from each other, which extend over a wide range   Structural chromosomal changes, also, are common   Relatively little is known however about human tumours as distinct from experimental animal tumours   In the face of the great variation which appears to occur, it is obvious that only by studying a large number of cases can any general pattern be expected to emerge   Only then may it be possible to discover whether or not a correlation exists between the chromosomal changes and the characteristics of the tumour, such as its degree of autonomy, its tendency to invade or metastasize, its degree of differentiation, or tendency to metaplasia, or its sensitivity to ionizing radiations

Studies on " ascites " tumours of rodents indicate that each tumour strain maintains, within limits, a degree of constancy, and has a modal chromosome number which may not change for many transplant generations , however the fact that changes may occur suggests that they may be a factor in the " progression " of tumours towards greater malignancy, or more specifically towards adaptation to changing environmental conditions   Unfortunately, relatively little is known about the chromosomes of primary (untransplanted) tumours, even in experimental animals   Perhaps the most urgent and intriguing problem is the relation of chromosomal change to the inception of the neoplastic process , nevertheless much may be learnt from the study of fully-developed tumours,

especially in man, where the findings may have a direct relevance to the clinical management of the condition

Several complementary approaches are often necessary in the attack on any single problem or group of problems   tissue-culture techniques, which have proved so useful in the study of the chromosomes of normal cells (Tjio and Levan, 1956 , Tjio and Puck, 1958) and of marrow cells in leukaemias (Ford, Jacobs and Lajtha, 1958 , Baikie, Brown, Jacobs and Milne, 1959), will no doubt be increasingly applied to the study of malignant disease, and much may be learnt   Apart from purely technical difficulties, however, the altered conditions in tissue-culture and the possibility thereby of the selection of certain cell-types from a mixed population may make it difficult to relate the findings to the state of the tumour before its removal from the body   In a previous paper (Atkin and Richards, 1956), we have discussed the application of microspectrophotometry to the study of human tumours   from an estimate of the amount of Feulgen stain, and hence of deoxyribonucleic acid (DNA), in the individual cells of a tumour, data can be obtained that give a measure of its chromosome complement   In a preliminary survey of normal tissues and malignant tumours from various sites, we were able to demonstrate that, while normal cells showed little variation from the diploid value, the interphase cells in the tumour specimens tended to show a greater range of DNA values, partly due to the presence of cells synthesizing DNA and partly to aneuploidy and polyploidy   Nevertheless the majority of cells were grouped around a modal value which varied in different tumours but in most cases was close to either the diploid or tetraploid level   Since the near-diploid tumours frequently had modes which were in fact a little above diploid, it was apparent that by this criterion (i e the modal DNA value) the majority of tumours differed from the normal   In a further study based on 14 cases in which we were able to compare directly DNA content and chromosome number for the same tumour (Richards and Atkin, 1960), it was found that the two sets of values were on the whole in agreement, although in several cases the modal DNA content was higher than would be expected if the ratio of DNA content to chromosome number which we had previously found for normal tissues still held   The ratio of DNA to chromosome number exceeded the normal value on an average by 14 per cent, with a range of −6 per cent to +47 per cent

In this paper are reported the data obtained by microspectrophotometry of Feulgen stain in a series of uterine carcinomata, which will be considered with special reference to their clinical and histopathological features , in a parallel paper (Richards and Atkin, 1959) the changes in DNA pattern that have been observed in some of these tumours during and following radiotherapy (including some radioresistant cases) are described

<center>MATERIALS AND METHODS</center>

(a) *Carcinoma of the cervix uteri* —Of 132 cases, biopsy material was obtained before treatment from 124, while from the remaining 8 cases specimens of local recurrences following radiotherapy only were measured   From 3 of the 124 cases measurements were obtained both from the tumour before treatment and from local recurrences which subsequently developed following radiotherapy   Thus the total number of locally recurrent tumours that were measured is 11

(b) *Carcinoma of the corpus uteri* —Measurements were obtained from 33 cases, of which 30 had not previously received treatment

All the material was obtained under general anaesthesia in the operating theatre, in the untreated cervix cases, biopsies were usually taken immediately before the first Stockholm insertion   Part of the material was retained for cytological studies, including, in suitable cases, chromosome counts, from the remainder, smears were prepared for subsequent measurement of Feulgen stain as previously described (Atkin and Richards, 1956)   At the same time, material was sent for routine histological examination   Although there may be relatively few late-stage cases, it is probable that the 124 cervix cases approximate to an unselected series, since biopsies were performed at this hospital on almost every case referred for treatment, including a number of Stage III and IV cases referred for palliative radiotherapy   A few cases (under 5 per cent of the total number) have been excluded from this series because of scarcity or absence of tumour cells in the biopsy material

In collating our results, derived as before from the measurement of the amount of stain in a random sample of interphase cells, we have been concerned with (a) the modal DNA value and (b) the variation within each sample   (a) *The modal DNA value* —In order to obtain a numerical value for the DNA mode, the following procedure has been adopted   the average value in arbitrary units of cells that fall within about 15 per cent of the mode is calculated   the data are plotted in the form of a frequency histogram with classes having a spread of about ± 5 per cent, and the 3 adjacent classes with the greatest number of cells are usually averaged   This value is then adjusted to that of the mean of the leucocytes and/or fibroblasts present in the specimen, which are given an arbitrary value of 100 units   this will be referred to as the *basic DNA value*   Since measurements on normal epithelial tissues, including cervical epithelium and endometrium (Atkin and Richards, 1956), have consistently been found to have modal DNA values about 10 per cent greater than the mean value of the leucocytes and other cells of mesothelial origin, it seems that the modal DNA value of epithelial tumours, calculated as explained above, should be related to a normal diploid value of 110, rather than to 100   (b) *The variation in individual samples* —Histograms of the DNA values of a number of individual uterine tumours have been given elsewhere (Atkin and Richards, 1956, Richards and Atkin, 1959)   In this paper we shall confine ourselves to some general observations on the degree of spread of DNA values in the tumour samples

RESULTS

We will first consider the data derived from the tumour samples obtained before treatment

(a) *The basic DNA values* —Excluding 2 cervical carcinomata, which are of special interest and will be considered later, all the tumour samples presented a clear mode   Fig 1 shows the principal modal, or " basic ", DNA value, calculated as described in the previous section, of 122 out of the 124 untreated cervical tumours, and of all the untreated corpus tumours   It will be seen that the cervical tumours fall into 2 distinct groups   Taking the value 156 as the upper limit of the lower group, there are 56 cases in the lower group and 66 in the upper   Furthermore it can be seen that when the logarithm of the modal DNA value is plotted, as in Fig 1   each group presents an approximately normal distribution   The lower group, which will be referred to as the ' lower ploidy group   has a rather narrower

distribution than the upper, with a coefficient of variation of 9 per cent, and a mean of 121 ± 11 It will be seen that this mean value is well above the mean leucocyte (*l*) value of 100, and about 10 per cent above the value (110) which, as already explained, we regard as the normal diploid epithelial value There are in fact only a few cases below 110 In contrast, the mean of the upper group (" upper ploidy group ") is close to the normal tetraploid epithelial value (220), the range is somewhat wider, and there are many hypotetraploid cases as well as some in the hexaploid region (mean = 223 ± 43 , coefficient of variation = 20 per cent) Although the numbers are fewer, the corpus cases present differences from the cervix cases which may be significant   there are relatively fewer tetraploid tumours and none in the hypertetraploid-hexaploid region , on the other hand, there are
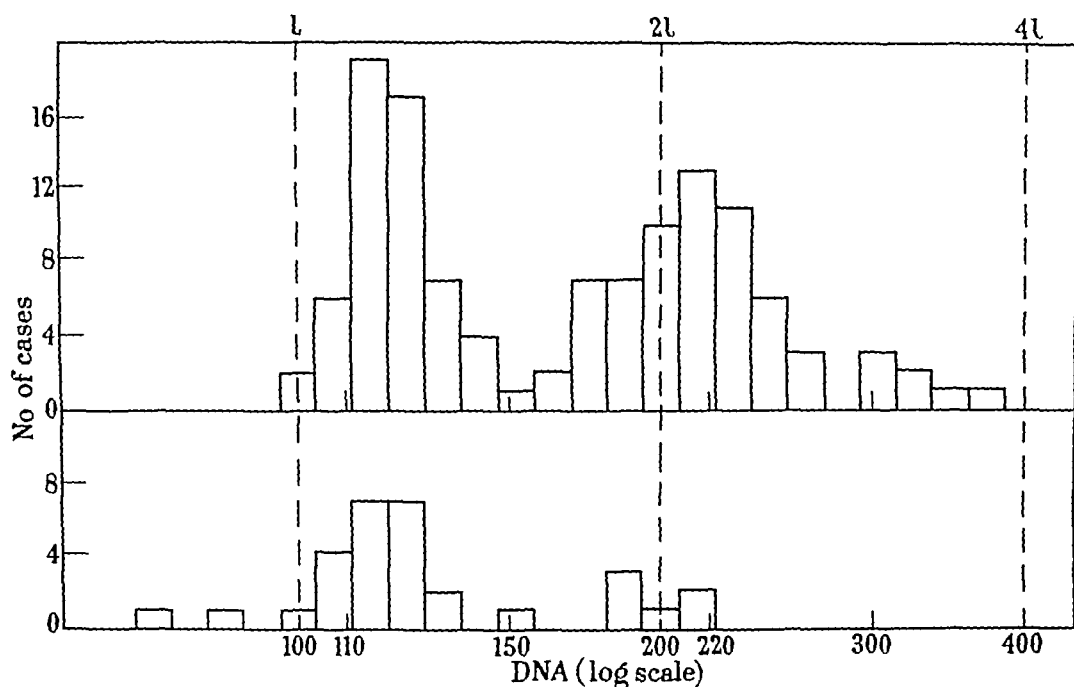
Fig 1 —Basic DNA value of 122 untreated cervical carcinomata (above), and 30 untreated corpus carcinomata (below)

2 hypodiploid tumours The mean of the lower ploidy group of the corpus tumours (neglecting the lowest value which is taken as being outside this range) is 118 ± 13 (coefficient of variation = 11 per cent)

The data are presented in tabular form in Table I In order to obtain an estimate of the number of tumours which fall close to the normal euploid levels, they have been classified according to whether they fall within ±15 per cent of the normal diploid epithelial value (110) or of twice this value Seventy-two per cent of the cervical tumours fall within one or other of these limits

(b) *The degree of spread of DNA values in individual tumours* —With a few exceptions, this is not very great   in the majority of cases from 50–90 per cent of the cells fall within ±15 per cent of the main mode There is usually a secondary mode at double the value of the main mode The relative height of this secondary mode varies in different tumours, reflecting the number of cells that have completed DNA synthesis prior to mitosis, or have achieved a doubling of the basic chromosome complement by abnormal mitosis, endomitosis or endoreduplication

Taking the 2 principal modes together, we find that in most cases from 70 to 9 per cent of the cells fall within ± 15 per cent of either of these limits To obtain a more precise estimate of the degree of spread, the results from the first 37 consecutive cervical cases were averaged, one case which had a wide spread being excluded 63 per cent of the cells fell within ±15 per cent of the main mode and 17 per cent within ±15 per cent of the secondary mode In comparison, 2 specimens of normal cervical epithelium and 2 of normal endometrium in the secretory phase showed 91–96 per cent of the cells within these two limits primary mode, 76–96 per cent secondary mode 0-17 per cent The degree of spread in the more undifferentiated tumours of the corpus uteri was similar to that in the cervix tumours but some of the well-differentiated corpus tumours showed less spread, the values falling within the range for the normal tissues just quoted In a few tumours which had a main mode in the tetraploid region or above, there was a smaller mode at about *half* the value of the main mode , the possible significance of this finding will be discussed later The cells that fell outside the two main modal ranges only rarely had values much below diploid In a few tumours, giant cells (i e cells having values of from octoploid to 32-ploid or higher) were fairly frequent

It is necessary to consider to what extent variations in the DNA of the cells, either as regards the position of the basic mode or the degree of spread, may occur in different regions of the same tumour, and therefore to what extent a small sample of tissue is likely to be representative of the whole Although some degree of variation in any given case cannot be excluded, the rather limited observations that have so far been made on samples from 2 or more regions of the same tumour have revealed no significant differences, except in the relative prominence of the primary and secondary modes

Having described the variations in DNA content in a series of untreated uterine tumours, we will try to assess the extent to which they can be related to the histopathological and clinical features of the tumours

TABLE 1 —*Untreated Cases Classified According to Basic DNA Value*

Lower ploidy group = up to 156 Upper ploidy group = over 156 Diploid group = 110 ± 15 per cent (94–126) Tetraploid group = 220 ± 15 per cent (187–253)

		Carcinoma of cervix	Carcinoma of corpus
Lower ploidy group	Hypodiploid	0	2
	Diploid	44	19
	Hyperdiploid	12	3
		56	24
Upper ploidy group	Hypotetraploid	10	1
	Tetraploid	45	5
	Hypertetraploid	11	0
		66	6
Wide range (diploid tetraploid)		2	—
		124	30

Histopathology

In Table II, the histological type of the tumours, classified as before according to the value of the basic DNA mode, is shown   It will be seen that there is no correlation between the DNA level and the degree of differentiation of the squamous cell carcinomata of the cervix, but that all the well-differentiated tumours of the corpus are diploid or hypodiploid   None of the adenocarcinomata or adenoacanthomata of the cervix, however, falls into the diploid or hypodiploid class   Thus there appears to be a significant difference between the squamous cell carcinomata of the cervix, which are frequently near-diploid, and the adenocarcinomata and adenoacanthomata, none of which has a near-diploid mode, although 2 of the adenoacanthomata have a wide range of values, including some in the diploid region   Table II shows that there are 45 squamous cell tumours in the diploid group and 70 in the higher-than-diploid groups, whereas the 17 tumours of the other two histological types are all above diploid or have no clear mode   This difference is highly significant ($p = 0\ 00054$), the limits that we have taken for the " diploid " group are purely arbitrary, but if we narrow the diploid group to ± 10 per cent or broaden it to ± 20 per cent, the differences are still significant ($p = 0\ 0020$ and $0\ 012$ respectively)   The adenoacanthomata of the corpus uteri, unlike those of the cervix, are all in the diploid class

There does not appear to be any correlation between the gross pathological type of the cervical tumours and the basic DNA value   Thus predominantly exophytic tumours occured in both the diploid and tetraploid classes

Clinical Features

(A) *Carcinoma of the cervix*

(1) *Age of patient* —The age-distribution of the lower and upper ploidy groups are compared in Fig 2   There are relatively more younger and older patients, as compared with those in the middle age-groups, in the upper ploidy group, espe-

TABLE III —*Carcinoma of the Cervix   Age of Patient, at Time of First Treatment, Compared with the Basic DNA Value*

		Under 45 years	45–64 years	Over 64 years
Lower ploidy group	{ Diploid	9	28	7
	{ Hyperdiploid	2	6	4
Upper ploidy group	{ Hypotetraploid	4	5	1
	{ Tetraploid	15	12	18
	{ Hypertetraploid	4	4	3
Wide range (diploid tetraploid)		0	2	0
		34	57	33

cially above the age of 74 where there are 11 cases as compared with 1 in the lower group   From Table III, it can be seen that there is a preponderance of cases in the middle age-groups (45–64 years) in the diploid class, but relatively fewer patients in these age-groups in the tetraploid class   These differences are not

TABLE II—*Histopathology of the Untreated Tumours and (in brackets) Those in which Specimens of Local Recurrences Only were Measured*

Degree of differentiation	Carcinoma of the cervix									Carcinoma of corpus					
	Squamous cell Carcinoma			Adenocarcinoma			Adenoacanthoma			Adenocarcinoma			Adenoacanthoma		
	Good	Moderate	Poor	Good	Moderate	Poor	Good	Moderate	Poor	Good	Moderate	Poor	Good	Moderate	Poor
Lower ploidy group { Hypodiploid	—	1	—	—	—	—	—	—	—	2	—	—	—	—	—
Diploid	2	7	35(1)	—	(1)	—	—	—	—	7	10	—	—	2	(1)
Hyperdiploid	1	3	7(1)	—	—	—	—	—	1	—	2	1	—	7	—
Upper ploidy group { Hypototraploid	1	5	27(1)	1	1	—	1	2	—	—	—	—	—	2	—
Tetraploid	3(1)	7	1(1)	—	2	2	2	1	1	3(1)	—	—	—	—	—
Hypertotraploid	—	—	—	—	—	—	—	2(1)	—	2(1)	—	—	—	—	—
Wide range	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—

statistically significant, however, and would have to be confirmed by observations on further cases
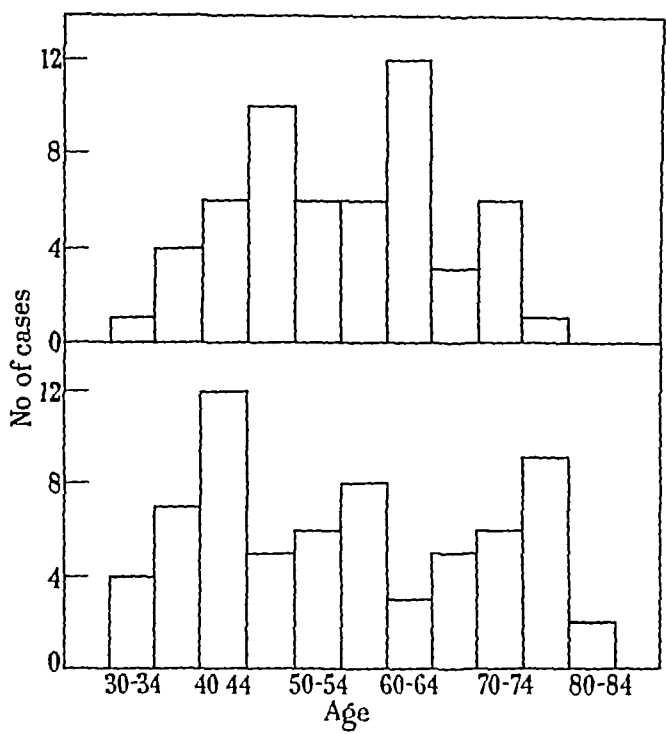
FIG 2 —Age distribution of 122 untreated cervical tumours   ABOVE   lower ploidy group
BELOW   upper ploidy group

(ii) *Clinical stage* —There appears to be no correlation between the clinical stage and the basic DNA value (Table IV)

TABLE IV —*Clinical Stage of Cervical Carcinomata*

		Clinical stage				
		I	II	III	IV	Total
Lower ploidy group	Diploid	14	15	10	5	44
	Hyperdiploid	2	5	4	1	12
		16	20	14	6	56
Upper ploidy group	Hypotetraploid	1	2	5	2	10
	Tetraploid	19	12	8	6	45
	Hypertetraploid	3	5	3	—	11
		23	19	16	8	66
Wide range		—	2	—	—	2
						124

(iii) *Response to treatment* —The great majority of cases were treated by a radical course of radiotherapy   either 3 radium insertions (modified Stockholm technique) or a single radium insertion supplemented by external irradiation with the 4 MeV linear accelerator   A few cases, mostly advanced, received external

irradiation with the linear accelerator or telecobalt unit only The average duration of follow-up, excluding 32 cases that have died, is 11 6 months, with a range of 0-52 months Of the 32 cases that have died, 11 were in the diploid class and 12 in the tetraploid Thus there appears to be no correlation between the DNA value and the survival rate, although most cases have only been followed up for a short time If however we consider those cases that developed a local recurrence in the cervix or vault of vagina, or were found to have viable tumour tissue in the cervix at subsequent operation, performed at least 21 months after the first treatment, we find that only one out of 11 had a diploid mode (Fig 3)
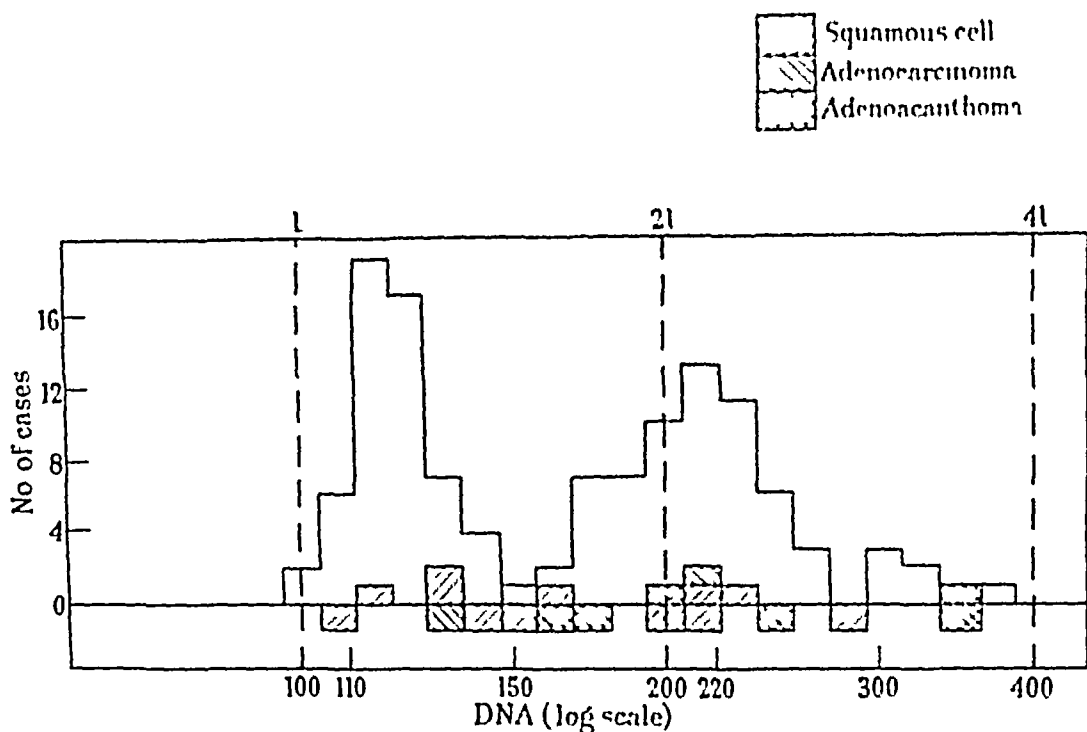
Fig 3 —Carcinoma of the cervix radioresistant tumours The basic DNA value of the untreated tumours which subsequently recurred locally after radiotherapy is shown (shaded) above the baseline , also, below the baseline are indicated the measurements made on local recurrences The continuous outline indicates the values obtained for *all* the untreated tumours

It was possible to obtain measurements on the local recurrences of 3 of these tumours, and of 8 further cases from which pre-treatment biopsies were not available The DNA values of these local recurrences are indicated below the base-line in Fig 3 When the data from these cases which showed a poor local response to radiotherapy are compared with the distribution of DNA values of *all* the untreated tumours (indicated by the continuous line in Fig 3), it can be seen that the former are more evenly distributed through the ploidy ranges, the majority being significantly greater than diploid The 2 adenoacanthomata that showed a wide spread of values before treatment (not indicated in Fig 3) gave rise to local recurrences both of which had modes in the triploid region histograms of these cases, including those derived from specimens obtained during the course of radiotherapy, are illustrated in the parallel paper (Richards and Atkin, 1959) Brief details of the cases that recurred locally are given below

Case No	Age Stage Degree of differentiation (histology squamous cell carc unless otherwise stated)—treatment (St = Stockholm radium insertion)	Basic DNA value before treatment	Subsequent history (figures indicate time in months after first treatment)	Basic DNA value of local recurrence
32	50 IV - Poor (adenoacanth oma) 1 St + DXR	197	No response 3 died	—
46	40 - I Poor (adenoacanth- oma) 3 St + DXR	—	8 local recurrence Further DXR (no response)	344
72	59 - II - Poor (adenoacanth- oma) 3 St + DXR	Wide range	Tumour failed to regress 2½ laparotomy Died shortly afterwards	161
78	52 II Good 3 St + DXR	—	2 local recurrence Wert heim's hysterectomy (died post operatively)	200
105	72 - IV - Poor (adenocarci noma of cervical stump) Intracav Ra + DXR	211	10 died , primary tumour still present	—
115	58 - III - Poor Intracav Ra (ovoids) + DXR	157	3 local recurrence , Wert heim's hysterect 27 symp tom free	—
319	51 - II - Poor 3 St	—	7 local recurrence , DXR Died shortly after	139
427	45 ? - Good (adenocarci noma) Total hysterec tomy followed by DXR to whole pelvis (4000 r)	—	7 tumour present in vault of vagina , further DXR Sub sequently died (tumour still present in vault)	127
480	40 - II Poor 3 St	133	8 cervix very hard (clinically recurrent growth, but biopsy negative) , external irradia tion (4 MeV linear accel) 15 deteriorating	—
502	66 - II - Poor (adenoacanth oma) 3 St	347	10 local recurrence , Ra needle implant—recurrence disap peared 16 mass in pelvis	242
537	47 - II - Poor (adenoacanth oma) 3 St	Wide range	Failed to regress 2½ positive biopsy , DXR 8 , died	168
548	39 - I Poor 3 St	—	5 local recurrence , Wert heim's hysterect 13 recur rent nodule in vagina , treat ment by 4 MeV linear accel	107
584	43 - I Poor 3 St	217	4 local recurrence , Wert heim's hysterect 10 vault recurrence and secondaries in scar , palliative DXR 12 deteriorating	—
585	61 - III Poor Telecobalt (7000 r)	117	6 mass side wall of pelvis— but cervix healed 10 local recurrence	—
703	45 I - Poor (areas of mod diffn) 3 St	228	4 cervix still bulky , Wert heim's hysterect (tumour cells present in cervix)	—
756	73 - II Moderate 1 St + radical DXR to pelvis	130	3 tumour still present in post fornix	—
824	53 - II - Poor 4 MeV linear accelerator	—	10 recurrence in cervix	208
825	32 - III - Poor 4 MeV linear accelerator	—	5 recurrence in cervix	148
833	60 - III - Poor 4 MeV lin ear accelerator	—	30 recurrence in cervix	277

(B) *Carcinoma of the corpus*

The age-distribution of the DNA classes for the 30 untreated cases is given in Table V Sixteen of these cases were treated by an intracavitary Co^{60} source (Strickland, 1953), and their subsequent history is given in Table VI Owing to

TABLE V —*Age-distribution of Untreated Cases of Carcinoma of the Corpus Uteri*

		Age													
		45-49	50-54	55-59	60-64	65-69	70-74	75-79							
Lower ploidy group	{ Hypodiploid	1	1												
	Diploid	2	1	1	1	1	1	1							
	Hyperdiploid		1			1	1								
Upper ploidy group	{ Hypotetraploid					1									
	Tetraploid			1	2	2									

TABLE VI — *Response to Treatment of 16 Cases of Carcinoma of the Corpus Uteri Treated by Intracavitary Co60 Source*

		Subsequent Wertheim's hysterectomy (1-3 months later) no tumour found on microscopic examination	No evidence of recurrence (no operation performed) Followed up for at least 2 years	Subsequent recurrence in pelvis	Not known
Lower ploidy group	{ Hypodiploid	2			
	Diploid	1	1	1	3
Upper ploidy group	{ Hypotetraploid	—		1	—
	Tetraploid	2		—	—

the small number of cases no deductions can be drawn from these figures, other than that both diploid and tetraploid cases may show a satisfactory response to this mode of treatment

Details of the 3 cases which had received previous treatment are given below

Case No 127 —Total hysterectomy in 1947, when aged 71, for moderately well-differentiated columnar cell adenocarcinoma Eight years later, polypoid mass in vault of vagina (histological appearance as before), treated by intracavitary radium with good response Basic DNA value 239

Case No 141 —Total hysterectomy followed by DXR to pelvis in 1953, adeno-acanthoma of moderate differentiation, aged 56 Two years later vault recurrence (similar histology), treated by intracavitary radium followed by total vaginectomy Died 6 months later secondaries in the abdomen, no tumour found in the pelvis at post mortem Basic DNA value 122

Case No 468 —Treated for carcinoma of the endocervix in 1954 by Stockholm radium technique moderately well differentiated columnar cell adenocarcinoma, then aged 76 In 1957, treated by total hysterectomy for ? recurrence ? new primary in body of uterus columnar cell adenocarcinoma, largely anaplastic, with a few areas of moderate differentiation—basic DNA value 225

DISCUSSION

The distribution of the basic DNA values of the cervical carcinomata (Fig 1) strongly suggests the presence of two distinct populations, it is reasonable to assume that the tumours in the higher group have undergone a doubling of their

chromosome complement at some stage of their evolution The central tendency in both groups is clear, and indicates that there is an *optimal region*, deviations from which are progressively less likely to occur in proportion to their magnitude The significance of the fact that the majority of tumours in the lower group are hyperdiploid rather than diploid as regards DNA content is not clear Data on stem-line chromosome counts collected by Ising and Levan (1957) suggest that human tumours in general are quite frequently hyperdiploid, and less often hypodiploid This is supported by our own somewhat limited observations on the chromosome numbers of the uterine tumours in the present series, counts in the range 48–52 being commonly found (unpublished data) On the other hand, Manna (1957) has found that many human cervical tumours have chromosome modes in the hypodiploid region It is concluded that more critical chromosome counts are necessary before we can assess the extent to which tumours with near-diploid basic DNA values do in fact differ from the normal in their chromosome complement If our finding (Richards and Atkin, 1960) of an average of 14 per cent more DNA per *chromosome in tumours* than *in normal tissues* were true *of* cervical tumours in general, we should expect the distribution of modal *chromosome numbers* of the near-diploid tumours to show a peak very close to the diploid number, and not in the hyperdiploid region However, our data, based on a small series of cases in which it was possible to compare DNA content with chromosome number for the same tumour, probably do not justify such a conclusion indeed they suggest that the discrepancy between DNA content and chromosome number is on the whole greater for the tumours with higher chromosome numbers, and minimal for the near-diploid group

The tumours in the upper group show a distribution that is centred on a value less than twice that of the lower group, and moreover have a rather wider range extending from the triploid to the hexaploid or hypo-octoploid region Does this represent a similarly wide range of chromosome numbers ? Bader (1959), in the course of microspectrophotometric measurements of DNA in a small series of human ovarian carcinomata, found in one case a discrepancy between the cells in anaphase, which fell into the diploid range, and those in interphase, which had a mode in the tetraploid region, and has suggested that tumours which are " tetraploid " as regards DNA content may not necessarily have a near-tetraploid chromosome number, since " a combination of diploid cells having doubled amounts of DNA and tetraploid cells having the tetraploid amount of DNA would result in a distortion of an interphase frequency distribution relative to the anaphase frequency distribution " We have already pointed out that some of our tumours, which have their main mode in the tetraploid or hypertetraploid region, have in addition a smaller mode in the diploid-hyperdiploid region Cytological studies on aceto-orcein squash preparations (Atkin and Ross, unpublished) indicate that these particular tumours are usually characterized by a high incidence of endomitosis, as also may be those that have a basically hyperdiploid mode plus a prominent hypertetraploid secondary mode , furthermore, although the chromosomes in these tumours are frequently crowded, sufficiently accurate counts have been possible to indicate that cells with near-diploid and near-tetraploid chromosome complements can reach an apparently normal metaphase stage It is not known, however, whether the tetraploid cells can complete mitosis It may be, therefore, that the DNA mode is not representative of the chromosome number of the stem-line in all cases , indeed, if there are *two* classes of *dividing* cells, one

having twice the chromosome complement of the other it seems quite likely that their relative frequencies cannot be deduced from the relative heights of the corresponding DNA modes However the tumours which have a lower secondary mode are in the minority (about 25–30 per cent of those tumours whose basic DNA value lies between 220 and 260) and for most tumours which are " tetraploid as regards DNA content the photometric findings are consistent with the cytological observations chromosome counts average nuclear size, and, where sex chromatin is evident, the presence of 2 sex chromatin bodies per nucleus (Atkin 1960)

The occurrence of endomitosis in the hyperdiploid-hypertetraploid group of tumours which includes those hypertetraploid tumours which do not have a hyperdiploid secondary mode suggests that these tumours may be in the process of or may have completed a transition from the lower to the higher ploidy Whether cells with a doubled chromosome complement can take over as a new stem-line will of course depend on whether they are capable of normal mitosis The greatest significance of polyploidy, however may be that it forms the basis for further evolution Levan (1956b) has pointed out that tetraploidy in mouse tumours is often only a transient stage on the way to hypotetraploidy secondary numerical variation is often superimposed on the chromosomal doubling Although this may well be true for human tumours also, there is no direct evidence for this at the moment The uterine tumours with hypotetraploid DNA modes do not show much endomitosis, nor do they have lower secondary modes , their mode of evolution is at present unknown

In view of the above considerations it is not surprising that no clear-cut correlation appears between the clinical and histopathological features of the tumours and their DNA values It might be that those tumours which deviate markedly from the diploid or tetraploid levels (i e those in the triploid or hexaploid region) could be regarded as genetically " unbalanced " and be expected more often to show anomalous behaviour However, we encounter the difficulty that their numbers are fewer and that they form a continuous series merging into the more nearly " euploid tumours so that any classification for statistical purposes must be purely arbitrary

The perhaps significantly higher incidence in the cervical tumours of high basic DNA values at the extremes of the age-range may reflect a greater tendency towards polyploidization, perhaps due to hormonal stimuli, in the younger and older patients as compared with those in the middle age-groups It is clear that there is no correlation in the squamous cell cervical tumours between the degree of differentiation and ploidy level This lack of correlation has also appeared in data (unpublished) on squamous cell tumours from other sites, including vulva, larynx and tongue, where well-differentiated tumours are relatively commoner On the other hand the figures for the corpus tumours suggest that here there may be a correlation between degree of differentiation and ploidy, since all the well-differentiated tumours are either diploid or hypodiploid , it may be that the degree of correlation between ploidy and differentiation varies in tumours of different histological type Perhaps therefore the finding that the adenocarcinomata of the cervix have predominantly high basic DNA values, in the small series of cases that we have observed, may be related to their more or less undifferentiated character rather then to any other factor

The adenoacanthomata of the cervix require further consideration None of

54

these had a diploid DNA mode, although before treatment two of them had a heterogeneous population (as regards DNA values) which included a number of cells in the diploid region   After irradiation therapy, however, in both these cases there emerged an actively-growing strain of cells having a triploid DNA mode   A further case, from which a pre-treatment specimen was not obtained, gave rise to a local recurrence which proved to have a main mode in the hexaploid region, with a smaller triploid mode   The rather frequent occurrence of triploid/hexaploid DNA modes in those adenoacanthomata which proved markedly radioresistant appears to be of particular interest   The possibility of a link between the histogenesis of these tumours, which has been discussed by Glucksmann and Cherry (1956) who also find that they frequently respond poorly to radiotherapy, their apparently anomalous DNA content and often radioresistant character would appear to merit further investigation *

The data from the squamous cell carcinomata of the cervix that subsequently recurred locally and the measurements actually made on local recurrences (Fig 3) indicate that these tumours are often higher than diploid, although the difference in distribution from that of the tumours which responded satisfactorily is not statistically significant   It is clear that there is no simple correlation between radiosensitivity and basic DNA level   Perhaps however with an increase in chromosome number there is the increased possibility of variation, which in some tumours may lie in the direction of greater radioresistance

SUMMARY

1   The DNA content of tumour cells from 132 cases of carcinoma of the cervix and 33 cases of carcinoma of the corpus has been estimated by microspectrophotometry

2   In all except 2 of the cervical tumours, a clear mode was apparent in the frequency distribution of DNA values of a random sample of interphase cells in most tumours, from 50 to 90 per cent of the cells fell within ± 15 per cent of a modal value, which has been referred to as the *basic DNA value*

3   The basic DNA values of individual tumours were found to extend over a wide range, but fell into 2 main groups centred on the hyperdiploid and tetraploid levels respectively   This distinction into 2 groups was clearly seen in the cervical tumours although individual tumours ranged from diploid almost to the octoploid level   There were relatively fewer corpus tumours in the tetraploid region and none with DNA values above tetraploid , on the other hand there were 2 hypodiploid tumours

4   The relation of the basic DNA value to the chromosome number of the tumour cells is discussed, and it is concluded that in the majority of cases the DNA value bears a reasonably close quantitative relationship to the modal chromosome number, although from evidence obtained in a previous study a strict parallelism is not necessarily to be expected   There is evidence that some of the cervical tumours may be in the process of transition from a hyperdiploid to a hypertetraploid DNA value by means of endomitosis or some other chromosomal-doubling process

* Measurements have recently been obtained on 3 further adenoacanthomata of the cervix Two had basic DNA values of 131 and 153 respectively before treatment   A specimen from the third case was not obtained before treatment, but one taken 7 days after the first Stockholm insertion had DNA modes in the triploid and hexaploid regions

5 There is no significant relation in the cervical tumours between basic DNA value and clinical stage or age of the patient although there is a suggestion that tetraploid tumours may be relatively commoner at the extremes of the age-range

6 There does not appear to be a correlation between the degree of differentiation of the squamous cell cervical tumours and their basic DNA value All of the 7 well differentiated corpus tumours however are either diploid or hypodiploid, but the number of cases is not large enough to demonstrate a statistically significant correlation There is a significant difference between the squamous cell tumours of the cervix which are frequently near-diploid, and the adenocarcinomata and adenoacanthomata of the cervix none of which has a near-diploid mode

7 Data derived from (i) untreated cervical tumours which subsequently recurred locally and (ii) measurements made on local recurrences suggest that radioresistant cell strains are more often higher than-diploid than near-diploid In particular several adenoacanthomata which responded poorly to radiotherapy had tetraploid, triploid or hexaploid modes, the two latter being relatively uncommon among the squamous cell tumours

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DNA CONTENT OF HUMAN TUMOURS CHANGE IN UTERINE TUMOURS DURING RADIOTHERAPY AND THEIR RESPONSE TO TREATMENT

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IONIZING radiations are widely used in the treatment of cancer in man and frequently they produce excellent results Sometimes, however, cases are encountered which give very poor results although they are of a type that can be expected to respond well to radiotherapy Although these cases are in the minority (a few per cent of the total) they remind us that response to radiotherapy can vary considerably between different tumours in any one histopathological group An additional complication is that the immediate response, viewed clinically or histologically, does not necessarily reflect the ultimate success or failure of the treatment These problems, both practical and biological, have been clearly annotated by Merrill (1958) in a review of the various attempts that have already been made to classify radiation response in terms of "radiosensitivity" and "radiocurability" A great need still exists, therefore, for an *objective* method for predicting radiosensitivity and radiocurability, the search for such a method is impeded in no small degree by the lack of understanding of the effects of radiation on the fundamental processes of dividing cells

For several years we have been measuring the DNA contents of human tumours both before treatment (which has formed the basis of a previous report (Atkin and Richards, 1956)) and in some cases during radiotherapy and, if possible, at later stages such as at operation Most of our data refer to uterine tumours partly because they are common and partly because the type of radiotherapy which they receive frequently makes it possible to obtain biopsies at several intervals during the course of treatment We have now collected sufficient results to warrant some account of the types of effect of the radiation treatment on the pattern of DNA values of the tumours and to consider these results with a view to obtaining a quantitative cytochemical method for assessing radiocurability

In addition to their practical interest, these observations are of value in the study of the effects of irradiation on a dividing cell population An extensive literature on this subject already exists but it contains few instances where the effects on the fundamental chemical processes, such as DNA synthesis, have been examined at the single cell level Our contribution is at a considerable disadvantage compared with an experimental study because we have no "controls", and the factors of the irradiation treatment are those dictated by the clinician In particular we must accept the fact that the dose received at any point in the tumour is difficult to assess and standardize Nevertheless, as we have previously suggested

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(Atkin and Richards 1956) the information that can be obtained from human tumours may be of more direct relevance to the study of this disease in man than perhaps much that is obtainable from studying transplantable tumours of animals The relative advantages of investigating spontaneous and transplantable tumours have recently been considered by Scott (1958)

MATERIALS AND METHODS

The details of our methods of preparation of tissues and measurements of Feulgen stain have been described previously (Atkin and Richards 1956) One improvement that has been made since that report is that the tumour material is now fixed by methanol freeze substitution at the hospital, thus avoiding the delay incurred in transport to King's College before fixation Feulgen staining is also done at the hospital and the Feulgen-stained specimens are transported mounted in non-drying immersion oil after having been dehydrated in the alcohol series and passed through xylene If necessary the stained preparations are stored for short periods at 2° C Immediately before measurement specimens are re-hydrated and mounted in glycerol This is necessary because the cell-crushing procedure which is part of the measuring technique, does not work satisfactorily with immersion oil as a mountant The amounts of Feulgen stain per cell nucleus were measured with the scanning photometer (Deeley, 1955)

The results for the DNA content (amount of Feulgen stain) for 60–100 tumour cells in each tumour are plotted as frequency histograms In some cases the scale of the amount of DNA is logarithmic instead of linear because some specimens, particularly after irradiation, show ranges of DNA values over several multiples of ploidy The method that we previously used for calibrating the amounts of stain so that different specimens may be compared, was also used here in this a sample of 10–30 inflammatory cells, usually polymorphonuclear leucocytes, is measured in every specimen these act as a standard which we designate the ' *l* ' value and the scale of amounts of DNA in the histograms is thus in terms of *l*

RESULTS

The limitations of any system of classification of human tumours are emphasized by the differences in response of the tumours in any one group to the same method of radiation treatment This variation in response between individual cases may appear in the early stages of treatment or only after completion of treatment Likewise, in the results reported here, the effect of irradiation on the DNA content and pattern of DNA values in tumours may vary between individual tumours in both early and late stages of treatment

The results described here are limited to those on tumours of the uterus for the reasons given in the Introduction In a parallel paper (Atkin, Richards and Ross (1959)) we have considered our results, both those presented here and from other tumours, in relation to the system of clinical staging that has been standardized for some years, and also to other factors, e g , age of patient In this report we have attempted to discuss the changes produced by the radiation treatment in the pattern of DNA values in all the cases in which we have been fortunate enough to have obtained specimens before, during and occasionally after the course of treatment Table I includes the relevant data on pathology and treatment for the cases which we have selected for illustration in this paper

TABLE I—*Table of Data for Cases Described*

Case No	Age	Site	Stage	Pathology	Treatment	Subsequent history	Fig No
501	48	Cervix	I	Poorly differentiated squamous cell carcinoma (P d sq c ca)	2 Stk * + Wert *	Died from widespread metastases 4 months later	1
491	51	Corpus	—	Moderately well differentiated columnar cell adenocarcinoma	1 Stk + total hysterectomy	Well 8 months later	2
161	73	,,	I	Moderately well differentiated columnar cell papillary adenocarcinoma	Intracav Co⁶⁰×2	Well 41 months later	3
421	43	Cervix	I	Moderately well differentiated keratinizing squamous cell carcinoma (oxophytic)	3 Stk	Well 19 months later	4
449	58	,, (stump)	II	P d sq c ca	Ra ovoids (60 mg × 30 hr) Wert	Well 9 months later	5
486	33	Cervix	I	P d sq c ca with slight keratinization	3 Stk	Well 21 months later	6
280	51	,,	II	Keratinizing sq c ca	Ra (modified Paris technique) + Wert	Recurrence left side of pelvis 12 months later	7
72	50	,,	II	P d adenoacanthoma	3 Stk + DXR to parametria	2 months later, colostomy (mass in pelvis and local recurrence) Died a few weeks later	8
537	47	,,	II	P d adenoacanthoma	3 Stk	2 months later at laparotomy inoperable tumour invading rectum course of DXR to pelvis Subsequently developed local recurrence in cervix and died 8 months after first treatment	9

* Stk = Stockholm radium insertion
* Wert = Wertheim's hysterectomy

The case have been grouped into [illegible] the type of change shown in the pattern of DNA [illegible]

1 Slight change

Although the conditions of irradiation of the tumours differed somewhat in detail according to the individual needs of the case the differences do not seem to be sufficient to explain the fact that 6 out of 29 cases show little, if any, early change in their pattern of DNA values, in contrast to the marked changes seen in the remaining cases.

An example of this type of case is given in Fig 1 (case No 504) which is a Stage I cervical carcinoma. The basic DNA value (i.e., the post telophase DNA value of most of the tumour cells which is usually indicated by a prominent mode near the lower limit of the histogram) is seen to be approximately the same for all three tumour samples. Although there are slight indications of change at day 7, such as a reduction in the height of the primary mode and increase in the frequency of higher DNA values, the pattern at day 28 is that of a typical dividing cell population. This impression is strengthened by the fact that all stages of mitosis were observed in parallel aceto orcein preparations. It is noteworthy, however,

that this case received only 2 Stockholm doses, the treatment being completed by operation

Fig 2 and 3 show two cases of carcinoma of the uterine body (case No 491 and 161) which were treated by radium and by cobalt (Co⁶⁰) insertions (Strickland,
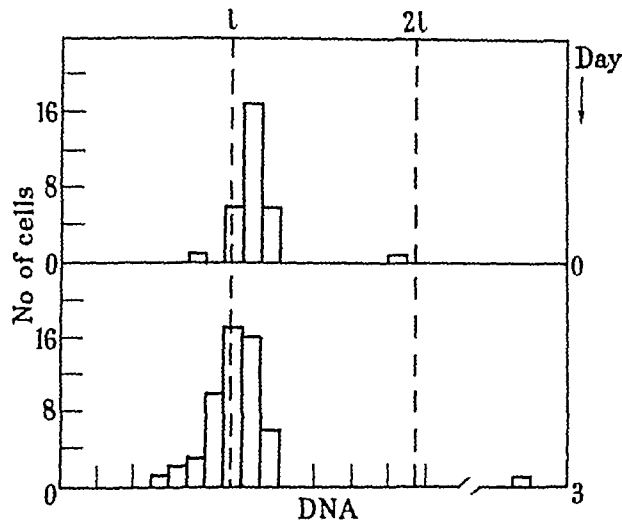
FIG 2 —Carcinoma of the corpus   before treatment , 3 days after radium insertion
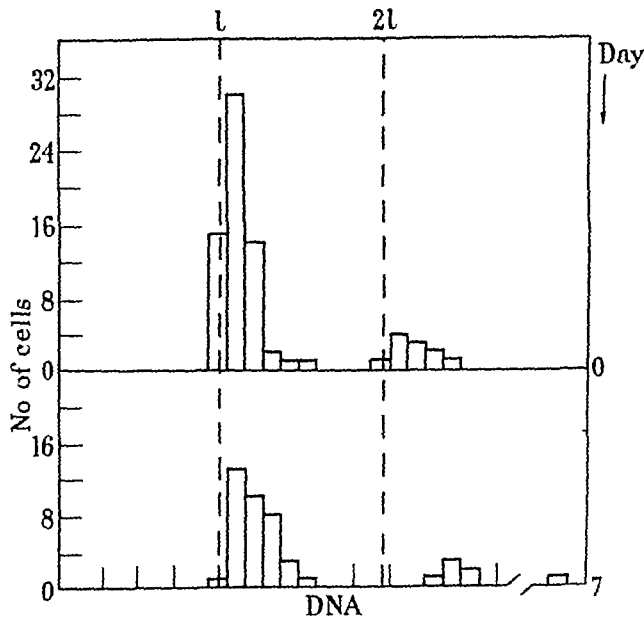
FIG 3 —Carcinoma of the corpus   before treatment , 7 days after first treatment with intracavitary Co⁶⁰

1953) respectively   In neither case can any significant change be detected at the short times after irradiation at which second specimens were available but in both these cases the specimen may have been taken too soon after the beginning of irradiation for marked changes to have occurred

Although all 6 cases, of which the aforementioned 3 are examples, failed to show significant changes in tumour samples taken during treatment, the clinical

response in each case was good, except in case No 501 where the presence of mitoses in the Wertheim specimen indicated that active tumour remained, but since 4 of these cases (including No 501) were operated upon (total hysterectomy or Wertheim's hysterectomy) at varying times following radiotherapy, the success of the radiation treatment alone could not be assessed

2 Typical changes

Apart from the 6 cases mentioned in the previous section, all cases studied showed significant changes in the early stages of treatment (i e at 7 days) These
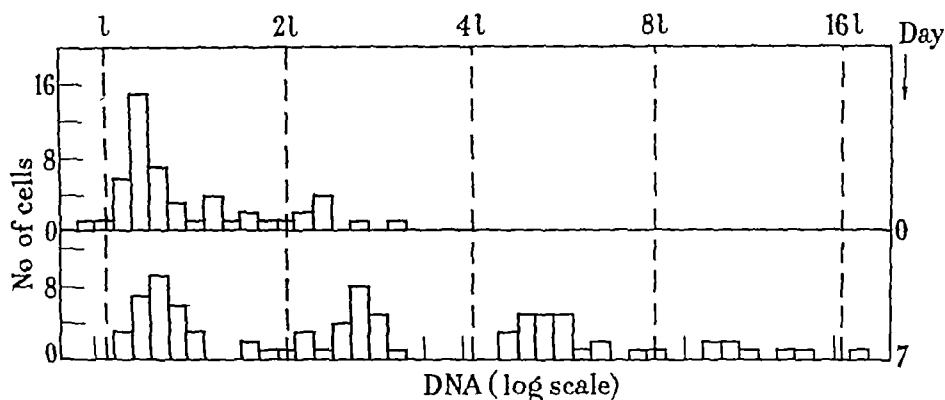
Fig 4 —Carcinoma of the cervix   before treatment , 7 days after first radium insertion
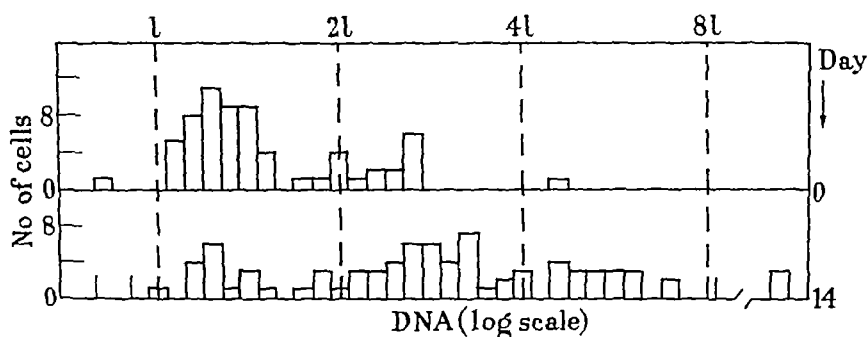
Fig 5 —Carcinoma of the cervix (stump)   before treatment , 14 days after first radium insertion

changes are regarded as typical of the early effects of irradiation on the pattern of DNA value because they appear in most tumours irrespective of additional or subsequent changes (or of the final outcome of the treatment) These changes are to some extent similar to those that have been observed as radiation effects on DNA content in the cells of transplantable animal tumours (see later)

Case No 421 (Fig 4), an exophytic Stage I cervical carcinoma, shows a striking accumulation of cells with larger DNA values, and, in this case, the histogram has definite modes at exact multiples of the basic DNA value   This is a characteristic pattern which is frequently seen at 7 days following the first radiation treatment These higher values are also commonly found at 14 days after a radium insertion, as for example, in Fig 5 (case No 449, a Stage II cervical tumour)   The appearance of higher multiples of DNA values is to be expected from the well-known " giant cell " formation after irradiation seen in histological studies

Both cases described so far in this section were near-diploid tumours, but the typical radiation response of increasing frequency of higher ploidy multiples is also found with near-tetraploid (2 *l*) tumours   An example of this is case No 486 (Fig 6), a cervical carcinoma, which at day 7 after the first radium insertion shows a prominent mode at near-octoploid (4 *l*) and also values in excess of 16 *l*   Seven days later (after a second insertion of radium at day 7) higher multiple values persist but there is some suggestion of a mode at the original basic DNA value

In all 3 cases so far described the last histogram represents the last occasion on which viable cells were present in samples taken during treatment , subsequent samples, where available, contained no tumour, and 6 months follow-up examina-
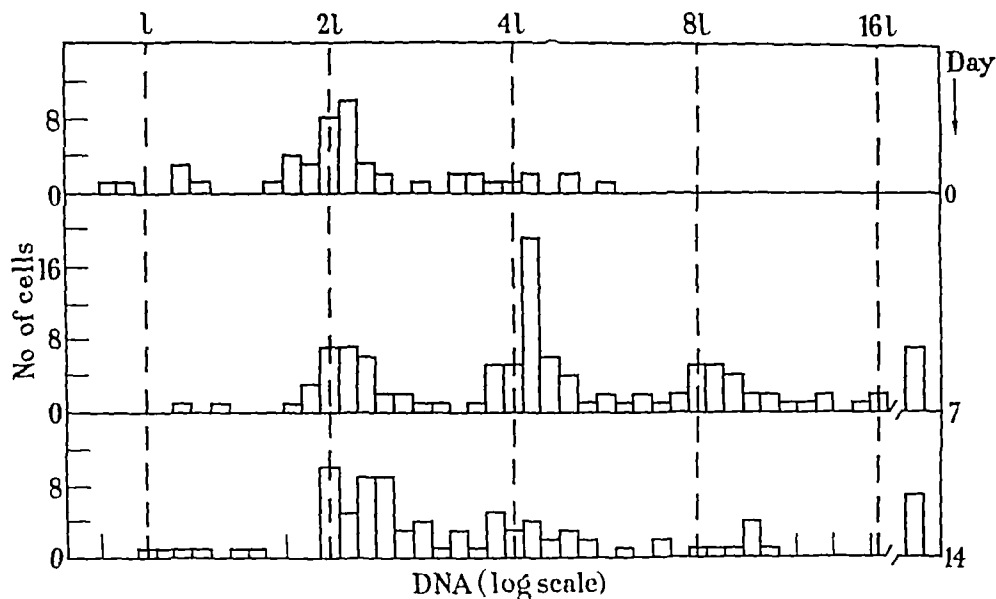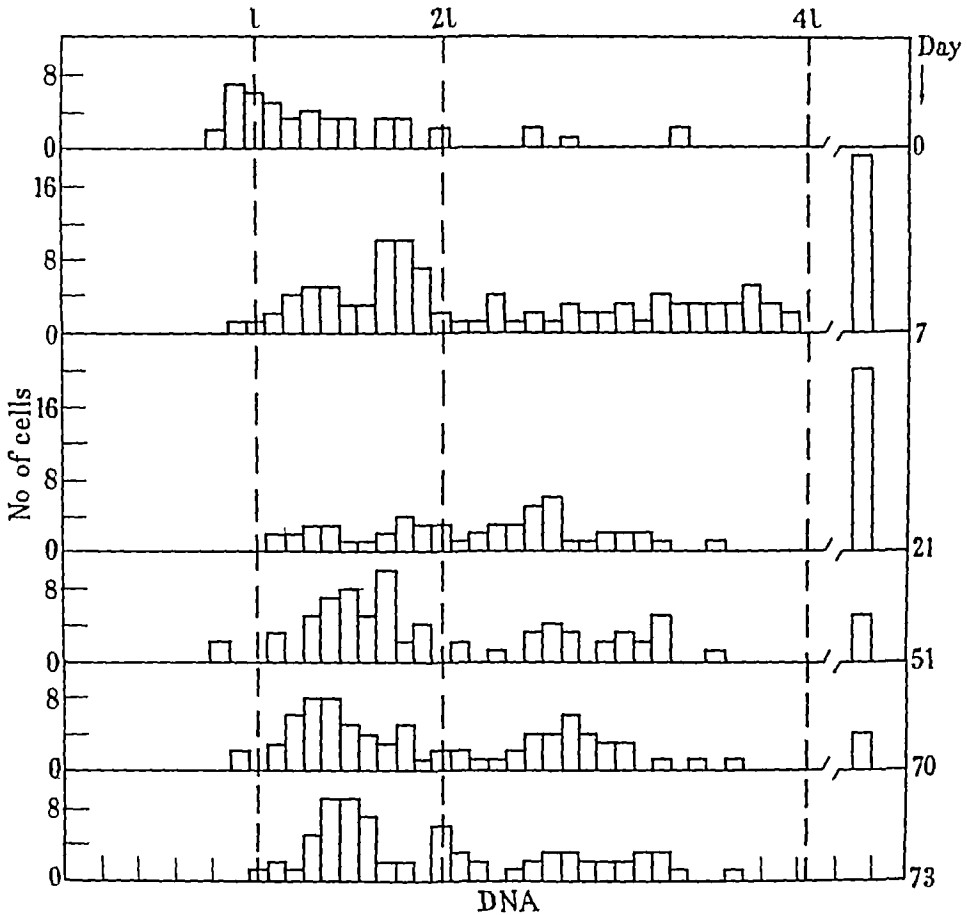

FIG 6 —Carcinoma of the cervix   before treatment , 7 days after first radium insertion , 7 days after second radium insertion

tions gave favourable results   The typical changes seen in the early stages of radiation treatment probably reflect only the sensitivity of the majority of the tumour cells to radiation and do not imply that the tumour as a whole is curable, because such response changes are found in both successfully and unsuccessfully treated cases

3 Unusual changes

Some of the cases that we have had the opportunity to examine have been noteworthy for one of two reasons   Firstly, they may have shown unusual features in the changes in their pattern of DNA values brought about by the radiation treatment, or secondly, they may have shown radioresistant properties which make them important from the clinical standpoint   Clearly, our aim is to see whether we find cases that are noteworthy for both reasons, or better still, if those that have a poor prognosis have patterns of DNA values *before treatment* which are unusual   In this way it might be possible to obtain an objective quantitative criterion of radioresistance   We shall now describe some of the cases in which

we have encountered unusual results and later attempt to assess the value of our observations with respect to such a criterion

An interesting case was one treated at the Middlesex Hospital by a modified Paris radium technique in which it was possible to obtain a biopsy very much sooner after the beginning of treatment than the customary 7 days (for the Stockholm method) This was a Stage II cervical tumour, the results for which
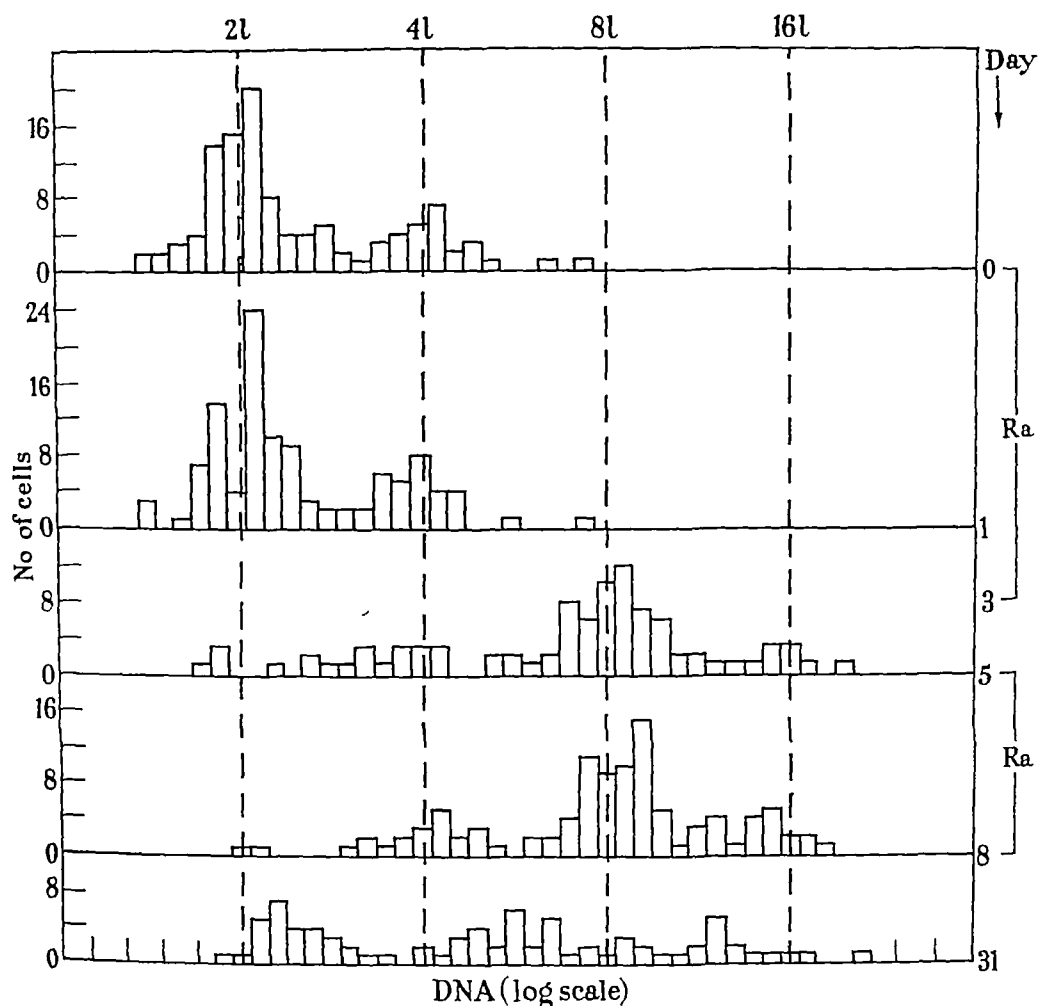
FIG 7 —Carcinoma of the cervix   before treatment , 24 hours after commencement of treat ment , 5 days after commencement of treatment , at completion of radiation treatment , at operation 31 days after commencement of treatment (radium was inserted for two periods of 3 days)

are illustrated in Fig 7 (case No 280)   Radium insertions were given for two periods   day 0 to day 3, and day 5 to day 8   It is noteworthy that the change in the pattern of DNA values towards the appearance of higher multiple values is not found at 1 day after the beginning of irradiation, whereas when 5 days have elapsed, during three of which the tumour was exposed to radiation, there is a very marked change in this direction   The patient underwent a Wertheim's hysterectomy at day 31 when the small residuum of tissue found indicated that the tumour was radiosensitive   After 4 months, however, a recurrence appeared in the left side of the pelvis

As already mentioned we have found two cases in which the radiation treatment seems to have had a more interesting effect on the tumour than the typical one of increasing the frequency of cells with higher DNA content, the typical effects in the early stages are nevertheless present The unusual feature is the fact that the pattern of DNA content in the tumour after the end of radiotherapy differs from that present in the untreated tumour in a manner not found in other cases The first of these is given in Fig 8 (case No 72—a Stage II cervical carcinoma)

FIG 8 —Carcinoma of the cervix (adenoacanthoma) before treatment , 7 days after first radium insertion , 14 days after second radium insertion , biopsy specimens of actively growing tumour at primary site obtained on days 51, 70 and 73

In this case, the pre-treatment biopsy is itself unusual with a relatively flat distribution , the basic DNA value is difficult to determine owing to the lack of a definite mode but is probably very near the l value The tumour failed to regress after radium treatment by a modified Stockholm technique so that tumour material was available at day 7 and 21 during radium treatment, at day 51 and 70 after treatment, and finally at day 73 when laparotomy was performed Soon after the last operation the patient died

The samples at day 7 and day 21 both show the typical increase in the frequency of higher DNA values of which, however, relatively few exceed 4 l , cell divisions were noted in each sample, and anaphases and telophases were seen at day 7 (i e , very soon after irradiation) Almost 2 months after the beginning of

treatment there was actively growing tumour at the primary site , this shows a fairly definite mode of DNA values at about 1½ l In both subsequent tumour samples this mode is very pronounced, and there is a range of values going up to but rarely exceeding 3 l This pattern of DNA values differs very much from that of the original tumour and hence it appears that a new actively growing tumour strain had emerged   The radiation treatment may have selected a resistant cell lineage having a different DNA content from the majority of the original tumour cells

Many of the unusual features of this case (No 72) were also found in another (case No 537—Stage II cervical carcinoma), the DNA values for which are given in Fig 9   Unfortunately, biopsies were available at day 0, 7 and 81 only   In the pre-treatment sample (day 0) an extremely wide range of DNA values is found,
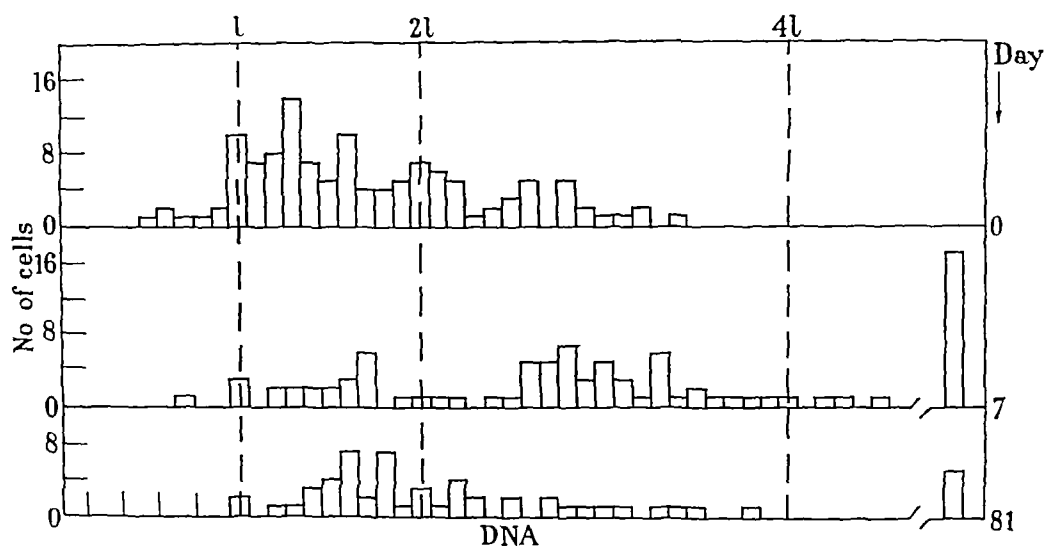
Fig 9 —Carcinoma of the cervix (adenoacanthoma)   before treatment , 7 days after first radium insertion , biopsy specimen of local recurrence on day 81

i e well outside the twofold range in which most of the cells of a dividing cell population usually lie   Like the previous case the pre-treatment distribution is essentially flat, but here the total range of values is much wider than twofold Although values near l are frequent, it is not possible accurately to assign a figure for the basic DNA value   Seven days after the first radium insertion the characteristic increase in the frequency of higher values has occurred but at day 81, when laparotomy was performed, there is evidence of a mode of values appearing between l and 2 l There are some indications, therefore, that the changes in the pattern of DNA values following treatment are similar to those found in the case last described, while a comparison of the clinical features and prognosis of the two cases reveals many similarities   Shortly after the end of radiation treatment a considerable amount of tumour remained   In this case (No 537) the tumour remained inoperable and, moreover, proved resistant to a further course of deep X-rays   It is interesting that both these tumours which had been dramatically unresponsive to radiation treatment and which showed a pattern of DNA values unlike that seen for other tumours, should have been identified as " mixed " tumours according to the histological classification of Glucksmann and Cherry (1956)   These authors also find such tumours to be frequently radioresistant